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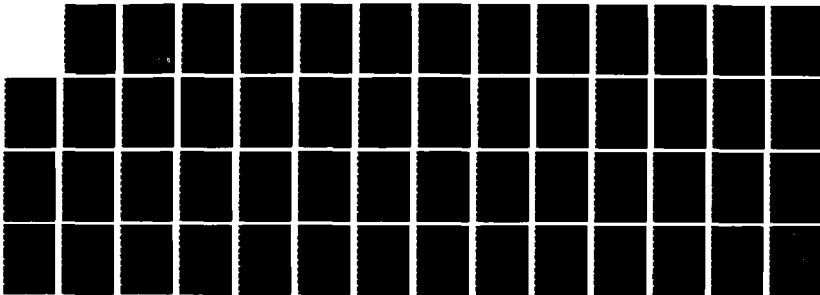
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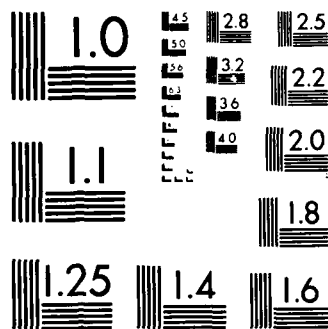
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THE DESIGN, SYNTHESIS AND SCREENING OF POTENTIAL
PYRIDINIUM OXIME PRODRUGS

Annual Report

Ronald T. Borchardt

July 31, 1985

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MARYLAND 21701-5012

Contract No. DAMD17-82-C-2078

University of Kansas
Center for Biomedical Research
Lawrence, Kansas 66044

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Pro-2-PAM has been synthesized and its biological properties studied. However, attempts to prepare other 3- or 5-substituted dihydropyridinium oximes (pro-2-PAMs) have not been successful.

To aid in the in vivo biological evaluation of potential regenerators of AChE, a simple and reliable high performance liquid chromatography (HPLC) assay for pyridinium oximes (e.g., 2-PAM) in biological tissue was developed. The assay is sufficiently sensitive to allow for detection of 2-PAM and the dihydropyridinium prodrug (pro-2-PAM) in brain and other biological tissues (e.g., kidney) and fluids (e.g., blood). Using this HPLC technique, we were able to demonstrate that a dose of pro-2-PAM delivers more 2-PAM to the brain than an equivalent dose of 2-PAM. Interestingly, we also observed significant brain levels of the pro-2-PAM, suggesting that the oxidation of the prodrug to 2-PAM in biological tissues is not as fast or as complete as previously assumed. These observations were confirmed with in vitro studies.

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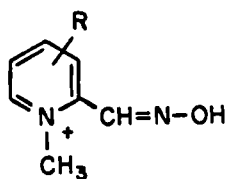
Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

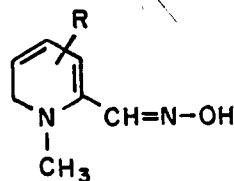
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Summary

In an attempt to improve the delivery of quaternary pyridinium oxime regenerators of acetylcholinesterase (AChE) to the central nervous system (CNS), structural analogs (I) and potential prodrugs (II) of N-methylpyridinium 2-carbaldoxime (2-PAM) have been synthesized. The potential prodrugs are dihydropyridinium oximes (pro-2-PAM's), which possess electron withdrawing substituents in the 3- or 5-position. As precursors to these prodrugs, we have synthesized and characterized this year a series of 5-substituted-2-PAM's (Cl, CH₃, CN, CONH₂ substituted) and a series of 3-substituted-2-PAM's (Br, Cl, CH₃ substituted). These new analogs and the 5-substituted 2-PAM's (I, Br substituted) and 3-substituted-2-PAM (I substituted) synthesized last year were tested in vitro for their ability to reactivate diisopropylfluorophosphate (DFP)-inactivated AChE, in vivo for their ability to protect mice from a challenge dose (2 X LD₅₀) of DFP, and in vivo for their ability to protect mice from a challenge dose (2 X LD₅₀) of soman.



I



II

The following order of potency was observed in the in vitro DFP-inactivated AChE assay: 3-chloro-2-PAM > 3-bromo-2-PAM > 3-methyl-2-PAM > 3-iodo-2-PAM > 5-methyl-2-PAM > 2-PAM > 5-chloro-2-PAM > 5-carboxamido-2-PAM > 5-bromo-2-PAM > 5-cyano-2-PAM > 5-iodo-2-PAM. When tested for their ability to protect mice from a challenge dose (2 X LD₅₀) of DFP, the following order of potency was observed: 5-iodo-2-PAM >> 3-iodo-2-PAM > 2-PAM > 3-bromo-2-PAM = 5-bromo-2-PAM > 5-carboxamido-2-PAM = 5-chloro-2-PAM >> 5-cyano-2-PAM. Many of these analogs have also been tested by the Walter Reed Army Institute of Research (WRAIR) for their ability to protect mice from a challenge dose of soman (2 X LD₅₀). 5-Iodo-2-PAM and 5-bromo-2-PAM were more effective than 2-PAM when administered with atropine. In the same test system 3-chloro-2-PAM and 3-bromo-2-PAM were less active than 2-PAM, whereas 3-iodo-2-PAM, 5-cyano-2-PAM and 5-carboxamido-2-PAM were inactive.

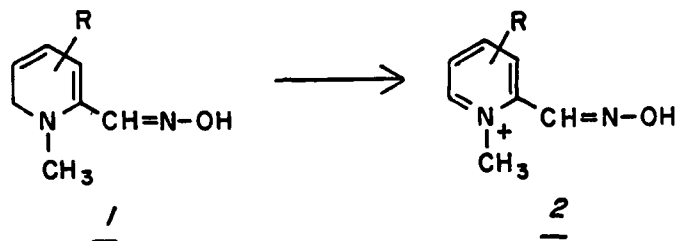
Pro-2-PAM has been synthesized and its biological properties studied. However, attempts to prepare other 3- or 5-substituted dihydropyridinium oximes (pro-2-PAM's) have not been successful.

To aid in the in vivo biological evaluation of potential regenerators of AChE, a simple and reliable high performance liquid chromatography (HPLC) assay for pyridinium oximes (e.g., 2-PAM) in biological tissue was developed. The assay is sufficiently sensitive to allow for detection of 2-PAM and the dihydropyridinium prodrug (pro-2-PAM) in brain and other biological tissues (e.g., kidney) and fluids (e.g., blood). Using this HPLC technique, we were able to demonstrate that a dose of pro-2-PAM delivers more 2-PAM to the brain than an equivalent dose of 2-PAM. Interestingly, we also observed significant brain levels of the pro-2-PAM, suggesting the oxidation of prodrug to 2-PAM in biological tissues is not as fast or as complete as previously assumed. These observations were confirmed with in vitro studies.

A. Problem

1. Design and Synthesis of Pro-PAM Agents

The overall objective of the project is to improve central nervous system (CNS) delivery of quaternary pyridinium oxime regenerators of acetylcholinesterase (AChE). The specific chemical problem is to design and synthesize nonquaternary, lipophilic prodrugs of pyridinium oximes which can be easily transformed in vivo into active quaternary regenerators. The prodrug forms consist of dihydropyridinium oximes 1a or the tetrahydropyridinium oximes 1b, which possess electron withdrawing substituents in the 3- or 5-position. Prodrugs 1b require elimination and oxidation to generate the corresponding N-methylpyridinium 2-carbaldoximes (2-PAM's) (2), whereas prodrugs 1a require only oxidation. It is hoped that the electron withdrawing substituents will stabilize the prodrugs, resulting in a slower in vivo conversion and thereby improvement of biodistribution to the CNS.



Determination of the rate and efficiency of conversion from prodrugs to parent pyridinium oximes will be required for evaluation of therapeutic potential and reactivator structural refinement. The measurement of physical properties such as pKa, partition coefficient and water solubility will be required to predict membrane penetration, tissue disposition and ability to dephosphorylate AChE.

2. Biological Testing

All drug candidates will require evaluation as regenerators of organophosphate-inhibited cholinesterases. Effective in vitro and in vivo screens will be necessary to evaluate both the parent pyridinium oximes and prodrug forms. In addition, a simple and effective pyridinium oxime detection technique for tissue distribution and elimination studies will be required. These studies will be essential in evaluating regenerator efficacy and CNS penetration.

B. Background

1. Regenerators

Organophosphates as a class owe their toxicity to their ability to react covalently with the esteratic site of AChE. AChE is the enzyme responsible for hydrolyzing the neurotransmitter acetylcholine. The phosphorylated enzyme can be reactivated by a variety of agents. There is, however, a competing "aging" process whereby the inactivating phosphoryl group either migrates to an adjacent amino acid residue¹ or is partially hydrolyzed.^{2,3} Phosphorylated AChE which has undergone this "aging" process is not easily reactivated. Thus rapid reactivation of the poisoned enzyme in all affected tissues is highly desirable.

I. B. Wilson discovered 2-PAM (3), which is now one of the most widely used and therapeutically effective broad spectrum AChE regenerators.⁴ Wilson postulated that the electrostatic attraction of the quaternary nitrogen helped orient the oxime moiety toward the phosphorylated esteratic site (Figure 1).^{5,6}

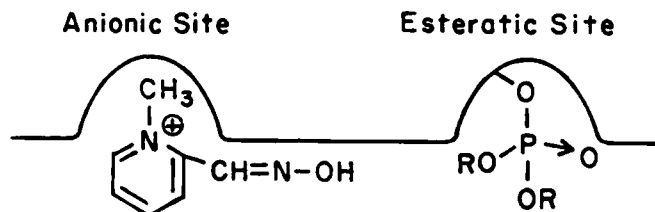


Figure 1. Proposed Active Site of AChE.

Since the discovery of 2-PAM, there have been a variety of other organic oximes which have been synthesized and screened for AChE regenerating activity (Figure 2). The charged bis-oximes trimedoxime (TMB-4) (4) and HI-6 (5) have both proven to be potent reactivators, but suffer from poor tissue penetration, short serum half-lives and toxicity problems.⁷ Neutral oximes such as monoisonitrosoacetone (MINA) (6) and 5-hydroxyiminomethyl-3-phenyl-1,2,4-oxadiazole (7) have shown much less potent regenerator ability, but do possess better lipid solubility.⁸⁻¹⁰

Investigations into the structure activity relationships of substituted 2-PAM's have produced some interesting information. In general, electron-withdrawing substituents shift the pKa of the pyridinium oxime to values below the optimum range of 7.4-7.8, and electron donating substituents shift it higher.^{4,11} Some 5-substituted 2-PAM's (Cl, CH₃) were approximately as effective as 2-PAM in whole animal survival studies, even though in vitro testing showed them to be less effective at regenerating deactivated AChE.¹²

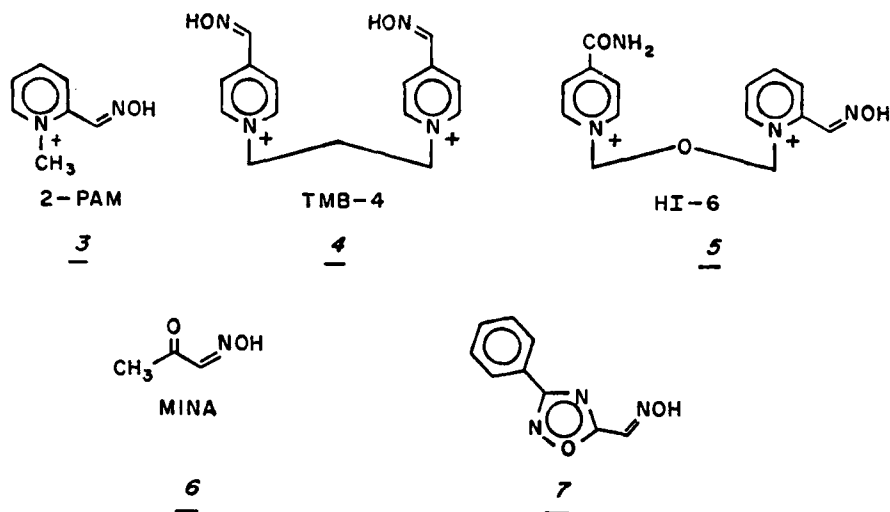


Figure 2. Regenerators of AChE.

These results indicate that the active site of AChE can tolerate minor structural changes in 2-PAM and, further, that desirable distribution characteristics might be incorporated into the molecule without significantly reducing its reactivating capabilities. In an attempt to increase the lipid solubility of 2-PAM, N-dodecyl-2-pyridinium carbaldoxime (2-PAD) was made. It possessed increased lipid solubility, but proved to be far less effective than 2-PAM at in vitro reactivation.¹³

Only trace amounts of 2-PAM can be detected in the CNS following i.v. injection.¹ Because of its high water solubility, the intact drug is rapidly eliminated from the body with an observed half-life in humans of less than 1 hour. Blood levels fall below the therapeutically effective range after 1-2 hours.^{14, 15} Significant brain levels of 2-PAM can be achieved only by intraventricular injections of the drug.^{16, 17}

Asphyxiation due to suppression of the central respiratory center is the ultimate cause of death in mammals exposed to anticholinesterase agents.^{18, 19} The lipid permeability of many organophosphates allows them to penetrate many body tissues, including the CNS, which are impermeable to a charged molecule like 2-PAM. Therefore, the need to regenerate AChE in the CNS presents an interesting drug delivery problem.

The delivery of 2-PAM to the highly lipid CNS was achieved with the pioneering work of Shek, Bodor and Higuchi.²⁰⁻²³ The University of Kansas group synthesized a prodrug of 2-PAM. Working on the hypothesis that a tertiary amine would have little difficulty penetrating the CNS, they synthesized a partially reduced form of 2-PAM which was a latent quaternary amine. Figure 3 illustrates how they trapped the reduced form of 2-PAM as a cyanide addition product, 9, which upon careful

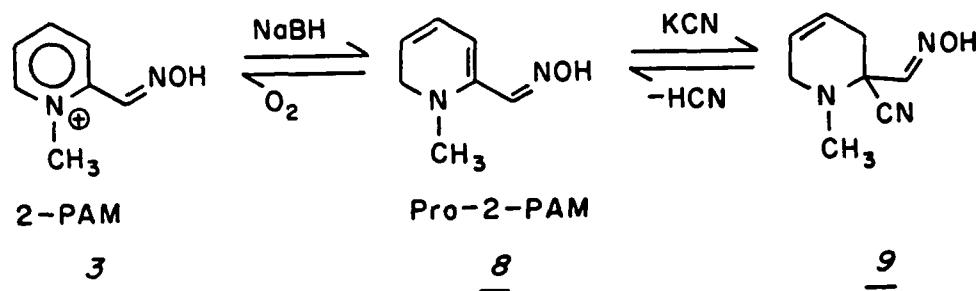


Figure 3. Synthesis of Pro-2-PAM.²⁰⁻²³

decomposition afforded pro-2-PAM (8). The pKa of 8 was determined to be 6.3, which was good for favorable physiological partitioning. They found that in vivo oxidation of 8 to 2-PAM (3), much like the NAD-NADH redox system, took place in approximately 1 minute.²² This was sufficient time to allow the drug to cross the blood-brain barrier, producing a 13-fold increase in brain levels of 2-PAM.²³

2. Biological Screening of Potential Regenerators

a. In Vitro Assays

Available in vitro screening techniques involve purified or partially purified soluble cholinesterase, which is exposed to an organophosphate and incubated with a regenerator, followed by the determination of enzyme hydrolytic activity. These assays generally involve the use of either acetylcholine or an analog which can be hydrolyzed by the enzyme. The resulting hydrolysis products can be measured colorimetrically, spectrally, and via pH change either directly or by CO₂ equilibria (both volumetric and radiometric).^{8, 9, 24-27} By far the most convenient and sensitive method is that developed by Ellman et al.,²⁸ which utilizes acetylthiocholine as substrate and measures the formation of thiocholine by its reaction with a disulfide chromogen.²⁸

b. In Vivo Assays

In vivo evaluation of oxime regenerators has employed mice, rats, guinea pigs and rabbits.²⁹⁻³¹ The determination of LD₅₀ values for potential regenerators as well as ED₅₀ values (vs. 2 X LD₅₀ challenge of organophosphate) are well documented and provide reliable, effective and comparable data. The determination of protective ratios (PR), in which doses of regenerators raise the lethal dose of organophosphates required to produce death, provides useful information about potential therapeutics.

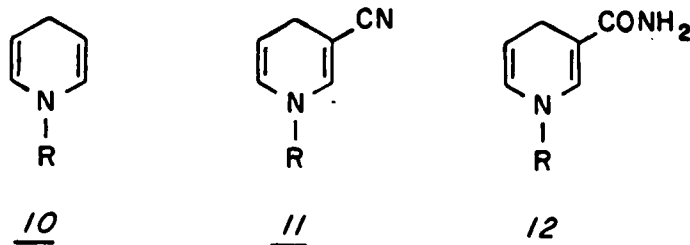
3. Biodistribution

Biodistribution patterns of pyridinium oximes have been determined, using three basic techniques; the administration of radiolabeled oximes followed by paper chromatographic identification is, to date, the most sensitive technique.^{23, 32} UV spectrophotometric quantitation of oximes in tissue extracts has also been employed; however,³³ this does not provide positive identification of the measured species. More recent developments involve the use of high performance liquid chromatography (HPLC) to identify and quantitate quaternary oximes in body tissues.³⁴

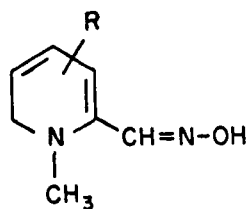
C. Approach

1. Prodrug Design

The basic approach has been to reduce measurably the rate of oxidation of a dihydropyridinium oxime to its active parent quaternary form by stabilizing the dihydropyridinium structure with electron-withdrawing substituents. It is known from the literature that an electron-withdrawing group in the 3- or 5-position stabilizes the dihydropyridines 11 and 12, relative to 10, through electron delocalization.



By synthesizing 3- and 5-substituted pro-2-PAM's 1, it is hoped



R = I, Br, Cl, CN, CONH₂, CH₃

that the rate of conversion from prodrug to the active quaternary pyridinium oxime forms can be slowed, thus allowing more time for partitioning into the CNS compartment (Figure 4). There may very well be a tradeoff

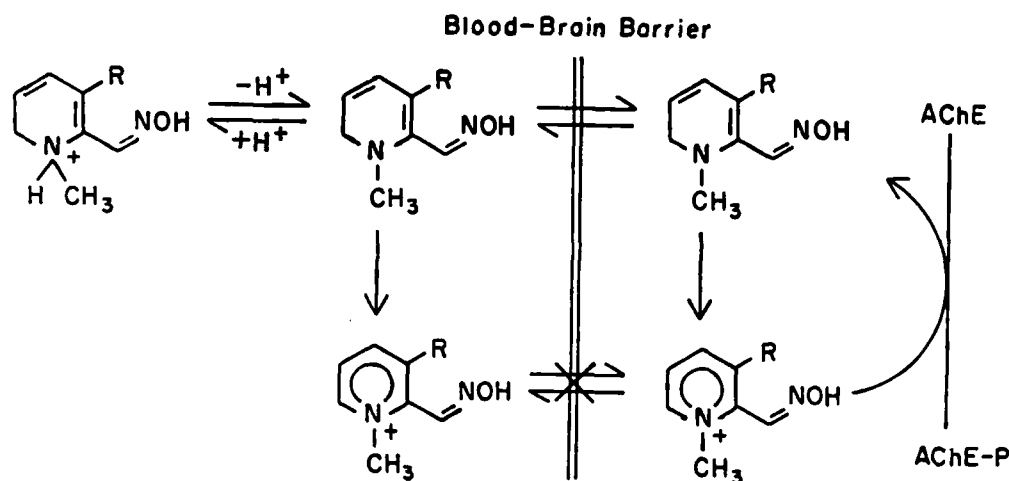


Figure 4. Pro-2-PAM Permeability of the Blood Brain Barrier and Conversion to 2-PAM.

2. Biological Evaluations

a. In Vitro

We have continued to employ our immobilized AChE assay³⁶ for screening the potential new regenerators being synthesized in our laboratory.

b. In Vivo

Working with mice, we have focused on the determination of LD₅₀ and ED₅₀ values (vs. 2 X LD₅₀ DFP challenge) of new regenerators. In addition, work on protective ratios should provide information helpful in evaluating new regenerators.

c. Biodistribution

Our efforts have focused on the development of assays, which allow for quantitative determination of tissue uptake and elimination patterns of pyridinium oximes. The use of HPLC and UV detection allows positive identification and quantitation of quaternary oximes and their metabolites in tissues. The examination of silicon-based, reversed-phase columns eluted with aqueous/organic solvents containing ion-pairing reagents for mobility control as well as hydrocarbon resin-based, reversed-phase columns eluted with aqueous solutions at various pH's for mobility control allows identification of several general, universally applicable conditions for identification and quantitation of pyridinium oximes in all tissues, especially the CNS.

D. Results and Discussion

1. Chemistry

In the previous annual report we described the synthesis of 3-iodo-2-PAM chloride (17m), 5-iodo-2-PAM chloride (17n) and 5-bromo-2-PAM chloride (17l), which were submitted to WRAIR for *in vivo* screening as regenerators of AChE. In this report we describe the successful synthesis of 3-methyl-2-PAM chloride (17a), 5-methyl-2-PAM iodide (16b), 3-bromo-2-PAM chloride (17c), 3-chloro-2-PAM chloride (17d), 5-chloro-2-PAM chloride (17e), 5-cyano-2-PAM chloride (17g) and 5-carboxamido-2-PAM chloride (17k). We have also described our unsuccessful attempts to prepare 3-cyano-2-PAM iodide or tosylate (16f), 3-carboxamido-2-PAM iodide or tosylate (16j) and the dihydropyridinium oximes 18g, 18k and 18n.

3-Methyl-2-PAM chloride (17a) and 5-methyl-2-PAM-iodide (16b) were prepared from 2,3-lutidine (13a) and 2,5-lutidine (13b), respectively, using the steps outlined in Figure 5. Sufficient quantities of 3-methyl-2-PAM chloride (17a) were prepared and sent to the Walter Reed Army Institute of Research (WRAIR) for *in vivo* testing. The synthesis of 3-bromo-2-PAM chloride (16c) was initiated with the bromination^{3/} of 2-picoline (19) to yield 3-bromo-2-picoline (13c) and 5-bromo-2-picoline (13l) (Figure 6). 3-Bromo-2-picoline (13c) was converted to the 3-bromopyridinium oxime 15c using the steps shown in Figure 5. Methylation of 15c with methyl iodide was unsuccessful; however, 15c was successfully methylated with methyl tosylate to yield the tosylate salt of 16c. The oxime 16c was converted in good yield to 17c using ion exchange chromatography (Dowex Cl⁻ form). Sufficient quantities of 3-bromo-2-PAM chloride (17c) were prepared and sent to WRAIR for *in vivo* testing.

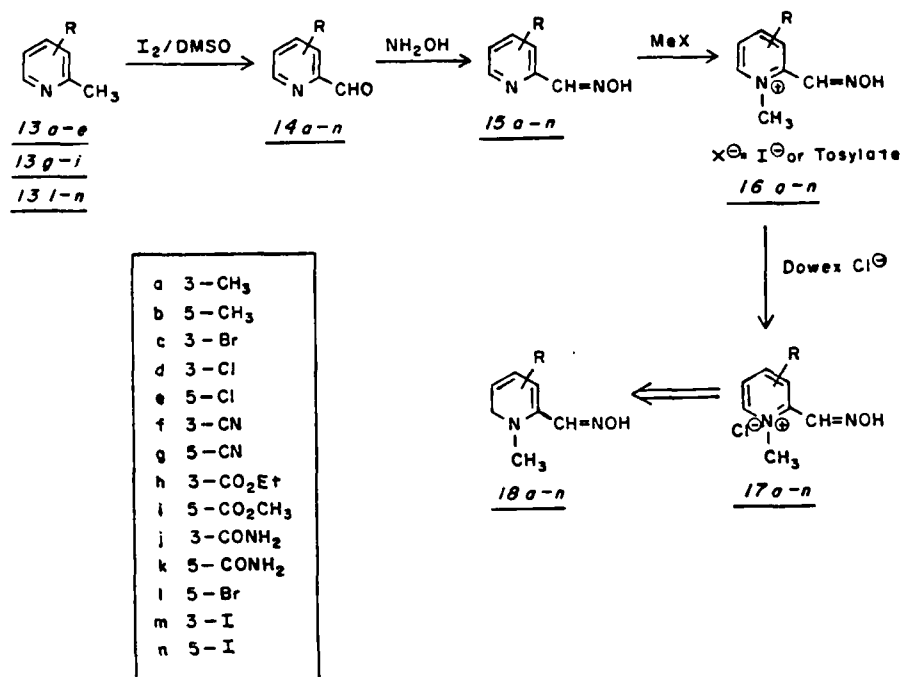


Figure 5. Strategies for Syntheses of 3- or 5-Substituted Pyridinium Oximes.

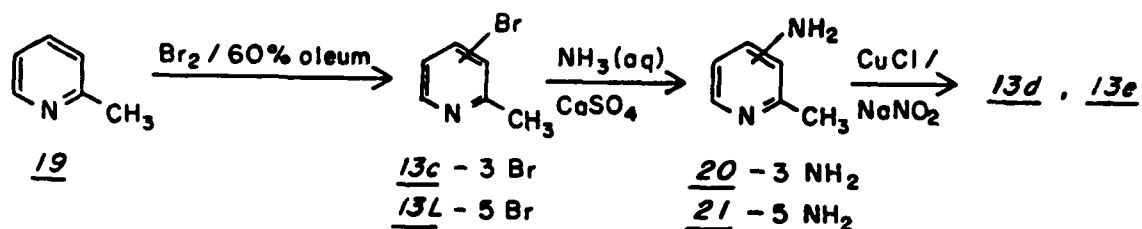


Figure 6. Syntheses of 3-Chloropicoline (13d) and 5-Chloropicoline (13e).

Attempts to prepare 3-chloro-2-picoline (13d) and 5-chloro-2-picoline (13e) by reaction of the corresponding bromo-picolines 13c and 13l with CuCl were unsuccessful. Attempted addition of methyl lithium to 3-chloropyridine also met with failure. However, a mixture of the chloropicolines 13d and 13e was prepared from a mixture of the aminopicolines 20 and 21, using the procedure to Talik *et al.*³⁸ (Figure 6). 3-Chloro-2-picoline (13d) was also prepared by reduction of 3,6-dichloro-2-picoline (25), using a modification of the procedure of Baumgarten *et al.*³⁹ 3,6-Dichloro-2-picoline (25) was prepared from 6-amino-2-picoline (22) as outlined in Figure 7.

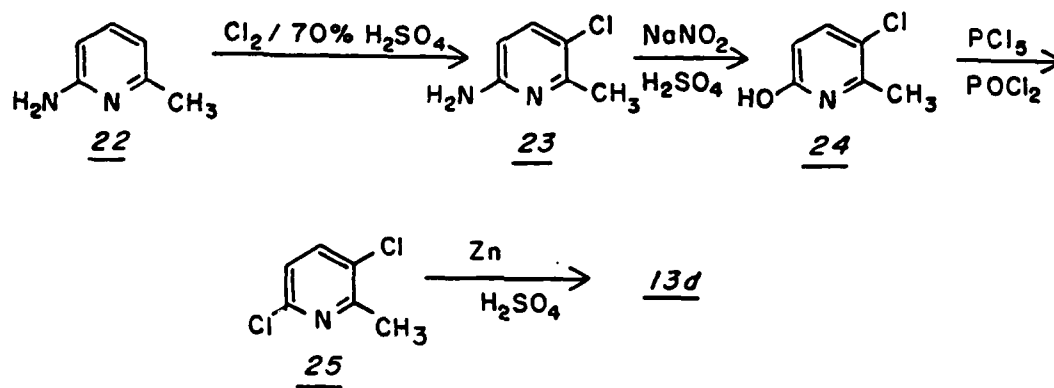


Figure 7. Synthesis of 3-Chloro-2-Picoline (13d).

In our previous annual report we described the syntheses of the 3-cyano aldehyde 14f and the 5-cyano aldehyde 14g from the corresponding 3-iodo aldehyde 14m and 5-iodo aldehyde 14n, respectively (Figure 8). As an alternative synthetic route to the 5-cyano aldehyde 14g, we started with 5-cyano-2-picoline (13g) and followed the synthetic steps outlined in Figure 5. The 5-cyano aldehyde 14g could be converted smoothly to

5-cyano-2-pyridine aldoxime (15g) in neutral aqueous hydroxylamine (Figure 8). Attempts to convert the 3-cyano aldehyde 14f to the aldoxime 15f using identical conditions resulted in decomposition of the starting material (Figure 8). However, the aldoxime 15f could be prepared directly in "one pot" reaction from the acetal 28 by reaction with aqueous HCl and NH_2OH . Attempts to methylate the 3-cyano aldoxime 15f with methyl iodide, dimethylsulfate or methyl tosylate in the presence or absence of K_2CO_3 lead to decomposition of the starting material. Attempts to methylate directly the 3-iodo acetal 26 and 3-cyano acetal 28 also met with failure.

Methylation of the 5-cyano aldoxime 15g with methyl tosylate afforded the desired 5-cyano-2-PAM tosylate (16g), which was converted to the chloride salt 17g using ion exchange chromatography (Dowex Cl^-) (Figure 5). Sufficient quantities of 5-cyano-2-PAM chloride (17g) were prepared and sent to the Walter Reed Army Institute of Research (WRAIR) for in vivo testing.

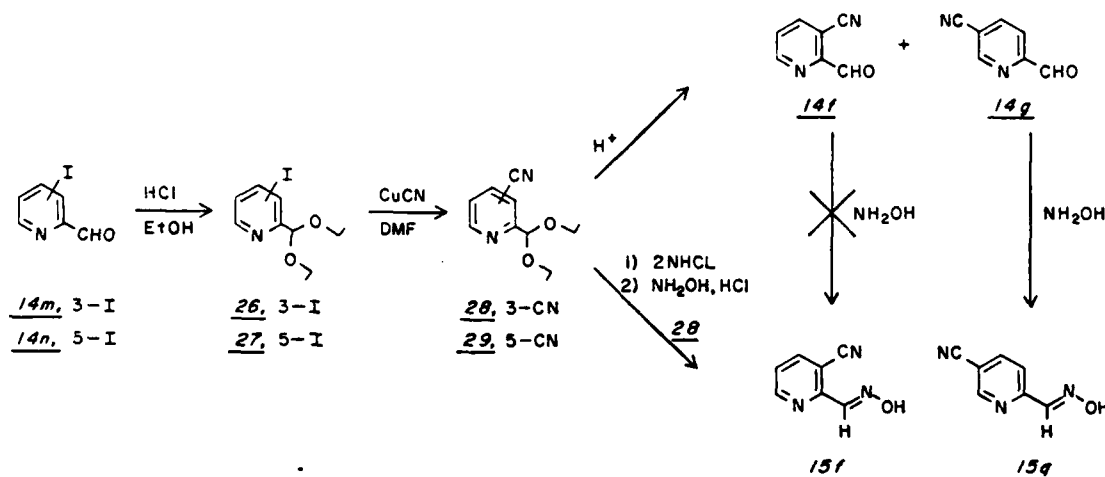


Figure 8. Syntheses of 3-Cyano (15f) and 5-Cyano (15g)-2-Pyridine Aldoximes.

In our previous annual report we described the synthesis of the 5-carboxamido oxime 15k from 5-cyano aldehyde acetal 29 (Figure 9). As an alternative synthetic route to the 5-carboxamido oxime 15k, we oxidized methyl-6-methylnicotinate (131) with I_2/DMSO to the corresponding aldehyde 14i. The aldehyde 14i was converted to the corresponding oxime 15i by treatment with NH_2OH . Aminolysis of 15i afforded the desired 5-carboxamido pyridine aldehyde 15k (Figure 9). The oxime 15k was methylated with methyl iodide to form the pyridinium aldoxime iodide 16k which was converted on ion exchange chromatography (Dowex Cl^-) to the chloride salt 17k (Figure 5). Sufficient quantities of 5-carboxamido-2-PAM chloride (17k) were prepared and sent to the Walter Reed Army Institute of Research (WRAIR) for in vivo testing.

In our previous annual report we had also described the synthesis of the acetal of 3-carboxamido aldehyde 30. Attempts to hydrolyze the acetal 30 to the aldehyde 14j resulted in the formation of 32 (Figure 9). As an alternative synthetic route to the 3-carboxamido aldehyde 14j, we oxidized ethyl-2-methylnicotinate (13h) with I_2 /DMSO to the corresponding aldehyde 14h. The aldehyde 14h was converted to the corresponding oxime 15h upon treatment with NH_2OH . Aminolysis of 15h afforded the desired 3-carboxamido pyridine aldehyde 15j (Figure 9). Attempts to methylate the aldoxime 15j using methyl iodide, dimethylsulfate and methyl tosylate failed.

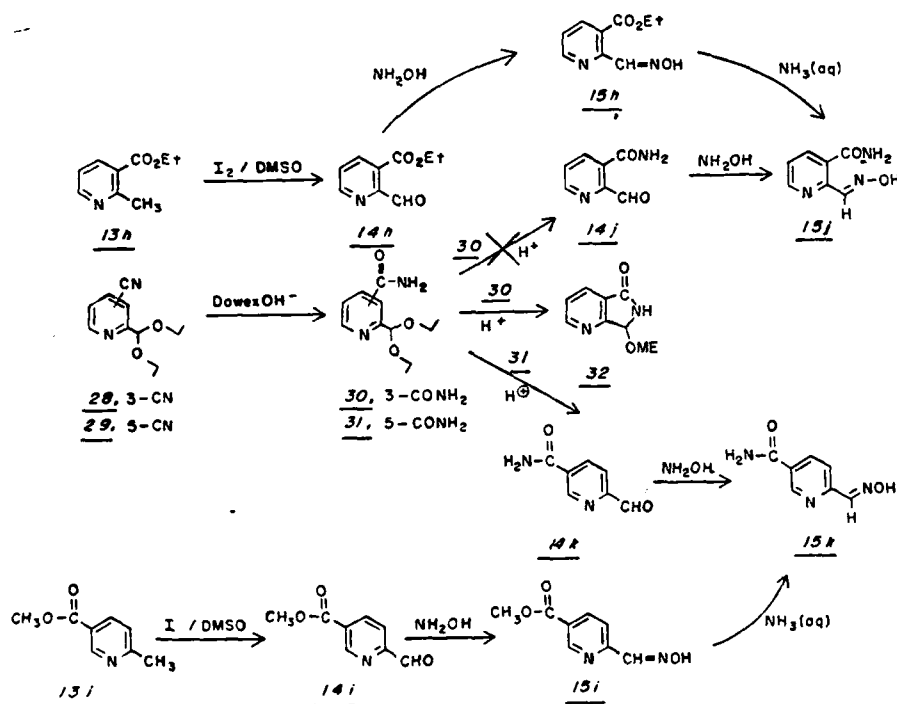


Figure 9. Syntheses of 3-Carboxamido (15j) and 5-Carboxamido (15k) Pyridine Aldoximes.

Following the literature procedures outlined by Bodor *et al.*²¹, we have prepared pro-2-PAM (8) from 2-PAM (3). This prodrug form of 2-PAM (3) was used in the biodistribution studied outlined in the Biological Results Section of this annual report. We have attempted to use the Bodor *et al.* procedure²¹ as outlined in Figure 10 to prepare 5-iodo-pro-2-PAM (18n), 5-cyano-pro-2-PAM (18g) and 5 carboxamido-pro-2-PAM (18k). However, reduction of the corresponding 5-substituted pyridinium oximes 17n, 17g and 17k with $NaBH_4$ in the presences of KCN did not afford isolatable and identifiable products. Therefore, we are forced to abandon this prodrug approach for substituted pyridinium oximes.

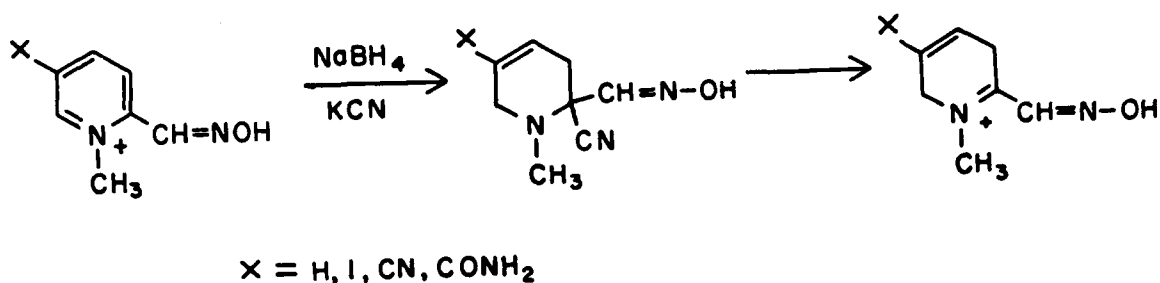
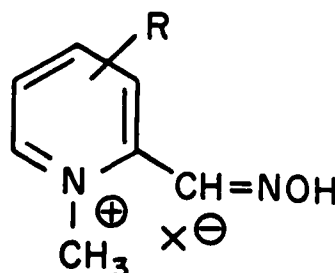


Figure 10. Attempted Synthesis of Dihydropyridinium Oximes.

2. Biological

Newly synthesized regenerators of AChE were evaluated in vitro, using immobilized AChE and the Ellman technique.^{28, 36} Eel AChE was attached via a stable Schiff base linkage to functionalized polyethylene beads using a modification of the procedure of Ngo, Laidler and Yam.⁴⁰ Acetylthiocholine was used as a substrate and DTNB as chromogen and the activity of AChE was followed spectrophotometrically at 412 nm.³⁶ A flow-through apparatus powered by a peristaltic pump was assembled, which allowed sequential and stepwise: (1) determination of initial immobilized enzyme activity; (2) inhibition with organophosphate; (3) reactivation with various oxime concentrations; (4) determination of regenerated enzyme activity; and (5) washing between steps to remove residuals. The ability of the 3- or 5-substituted 2-PAM's to regenerate DFP-inhibited AChE is summarized in Table 1. It is interesting to note that all of the 3-substituted 2-PAM's (3-iodo, 3-bromo, 3-chloro, 3-methyl) were as potent as regenerators of DFP-inactivated AChE than 2-PAM. In contrast, the 5-substituted 2-PAM's were in general less effective in regenerating the inactivated enzyme.

Table 1. Effects of 3- or 5-Substituted 2-PAM's as Regenerators of DFP-Inactivated AChE.

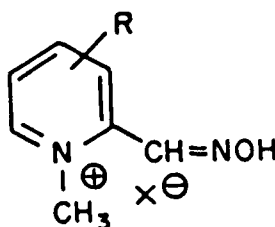


Compound	R=	Abs. % Reactivation ^a	% Reactivation Relative to 2-PAM
2-PAM	H	53	100
3-iodo-2-PAM	3-I	64	121
5-iodo-2-PAM	5-I	25	47
3-bromo-2-PAM	3-Br	77	145
5-bromo-2-PAM	5-Br	37	70
3-chloro-2-PAM	3-Cl	88	166
5-chloro-2-PAM	5-Cl	49	92
3-methyl-2-PAM	3-CH ₃	69	130
5-methyl-2-PAM	5-CH ₃	57	107
5-cyano-2-PAM	5-CN	28	53
5-carboxamido-2-PAM	5-CONH ₂	36	70

^aImmobilized eel AChE was inhibited with DFP (6×10^{-4} M), then washed free of excess organophosphate.³⁶ The DFP-inactivated AChE was incubated with 10^{-3} M oxime at 37° for 5 minutes. After exposure to the oxime, the enzyme was washed free of regenerator, and AChE activity measured.

Experiments were also carried out to determine the ability of the 3- or 5-substituted 2-PAM's to save mice challenged with DFP. Following a dose (sc) of $2 \times LD_{50}$ of DFP, mice were immediately administered (im) various doses of the 3- or 5-substituted 2-PAM's. The results shown in Table 2 indicate that the most potent analog in vivo against a DFP challenge is the 5-iodo-2-PAM. However, 3-iodo-2-PAM, 3-bromo-2-PAM and 5-bromo-2-PAM also showed significant activity. The potent activity of the 5-iodo-2-PAM and 5-bromo-2-PAM have been confirmed through independent studies in soman-challenged mice conducted by personnel at the Walter Reed Army Institute of Research (WRAIR).

Table 2. ED₅₀a Values for 3- or 5-Substituted Pyridinium Oximes and 2-PAM in DFP-Challenged Mice.^b



Compound	R=	ED ₅₀	
		μmol/kg (95% C.L.)	mg/kg (95% C.L.)
2-PAM	H	46 (62-35)	7.9 (11-6)
3-iodo-2-PAM	3-I	30 (40-22)	8.8 (12-6.7)
5-iodo-2-PAM	5-I	1.2 (2.3-0.6)	0.35 (0.69-0.18)
3-bromo-2-PAM	3-Br	59 (77.5-44)	14.9 (19.5-11)
5-bromo-2-PAM	5-Br	58 (88-39)	14.7 (22-9.8)
3-chloro-2-PAM	3-Cl	211 (237-188)	44 (49-39)
5-chloro-2-PAM	5-Cl	225 (267-191)	46.5 (55-39.5)
3-methyl-2-PAM	3-CH ₃	252 (325-196)	47 (61-36.5)
5-methyl-2-PAM	5-CH ₃	187 (224-156)	35 (42-29)
5-cyano-2-PAM	5-CN	—	—
5-carboxamido-2-PAM	5-CONH	105 (122-88)	22.6 (26.3-19.0)
pro-2-PAM ^c		117 (186-73)	20.3 (32-13)

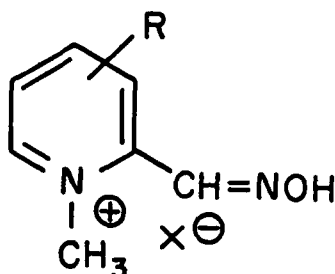
^aOxime dose required to obtain 50% survival at 24 hr.

^b(2 X LD₅₀, s.c., water), oxime (i.m., water, 1 ml/kg inj. vol.).

^ci.v., 50 mM citrate, pH 3.5.

In an attempt to correlate the in vitro and in vivo biological effects of the 3- or 5-substituted 2-PAM's with some physico-chemical parameter, we have determined the partition coefficients (octanol/water) and the pK_a values for the oxime functionality. The results of these experiments are shown in Table 3. It should be noted that 5-iodo-2-PAM and 5-bromo-2-PAM, which were the most potent analogs in soman-challenged mice, also have octanol/water partition coefficients which would favor CNS permeability and the pK_a values for the oxime functional group are in an acceptable range for optimal reactivation of organophosphate-inactivated AChE.

Table 3. pKa and Partition Coefficients for 3- and 5-Substituted 2-PAM's.



Compound	R=	pKa ^a	Octanol/H ₂ O Partition Coeff. ^a
2-PAM	H	7.9	0.0012
3-iodo-2-PAM	3-I	7.8	0.01
5-iodo-2-PAM	5-I	7.5	0.01
3-bromo-2-PAM	3-Br	7.8	0.004
5-bromo-2-PAM	5-Br	7.5	0.01
3-chloro-2-PAM	3-Cl	7.9	0.005
5-chloro-2-PAM	5-Cl	7.6	0.004
3-methyl-2-PAM	3-CH ₃	8.3	0.001
5-methyl-2-PAM	5-CH ₃	8.2	0.0004
5-cyano-2-PAM	5-CN	6.8	0.002
5-carboxamido-2-PAM	5-CONH ₂	7.3	0.0002
pro-2-PAM		6.3, 10.5	0.45

^aSee Experimental Methods Section for experimental details.

In an effort to expand the in vitro testing capability in our laboratory, we have immobilized on functionalized polyethylene beads human RBC AChE. The immobilized RBC AChE exhibited distinct differences from the immobilized eel AChE in response to organophosphates (DFP, paraoxon, soman). As shown in Figure 11, the RBC AChE was less sensitive than the eel AChE to soman at concentrations greater than 5×10^{-9} M. The RBC AChE was slightly more sensitive to DFP than eel AChE at all concentrations tested. Against DFP inactivation, RBC AChE was less reactivated (26%) than eel AChE (53%) by 2-PAM (10^{-3} M) during a 5 minute incubation. We also observed that the DFP-inactivated RBC and eel enzymes underwent aging at approximately the same rates (0.57% activity loss and 0.41% activity loss, respectively).

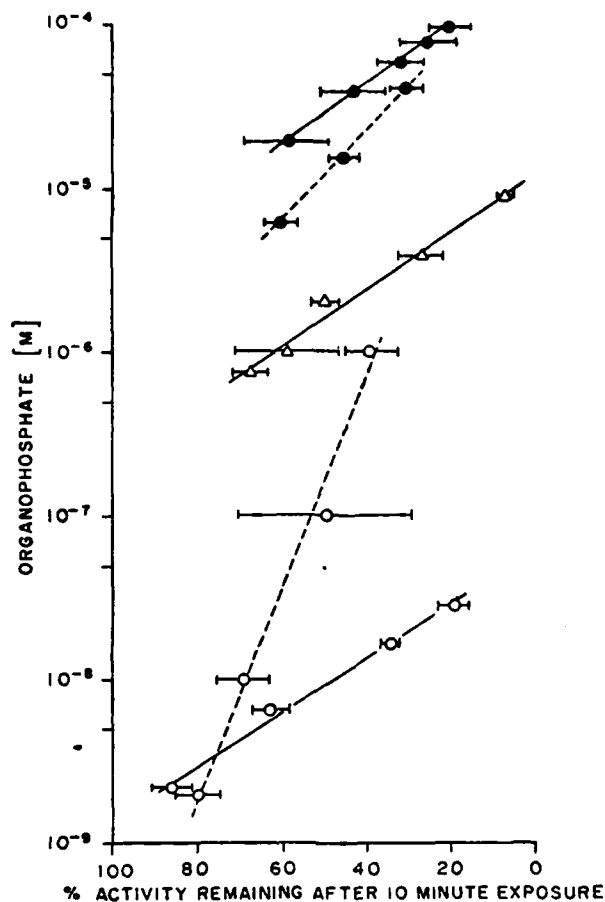


Figure 11. Inactivation of Immobilized Human RBC and Eel AChE to Organophosphates.
 Eel AChE (—○—), RBC (---○---), DFP (●), Paraoxon (△) and Soman (○). See Experimental Methods for experimental details.

Our laboratory has developed a new and efficient HPLC assay for regenerators of AChE. This assay has been used to monitor the tissue levels of both 2-PAM and its prodrug form, pro-2-PAM. The HPLC systems developed in this study utilize a commercially available, polymer-backed, reversed-phase column that allows the use of both strongly acidic and strongly basic buffers (pH range 1 to 13). Figures 12 and 13 show that these HPLC systems produce separations that allow for detection and quantification of 2-PAM or pro-2-PAM in biological tissues.

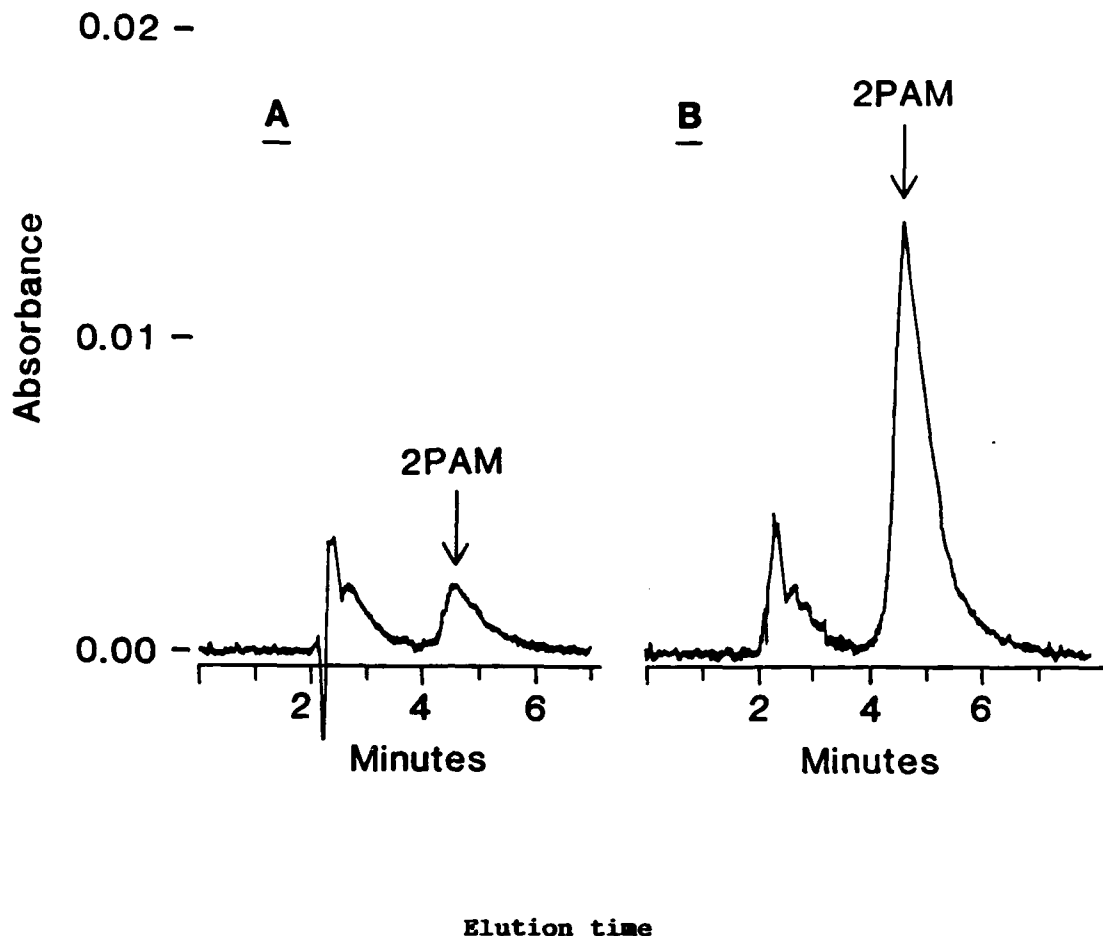


Figure 12. HPLC Elution Pattern of 2-PAM.

Typical elution profiles of 2-PAM from a dose of 2-PAM (A) (i.m., 50 mg/kg) and 2-PAM from a dose of pro-2-PAM (B) (i.v., 50 mg/kg) in brain tissue samples using a basic solvent system (100% Na_2CO_3 buffer, 0.1 M, pH = 10.5) and chromatography on a PRP-1 reversed-phase column. 20 μl injection; flow rate = 1.0 ml/min; detector wavelength = 334 nm.

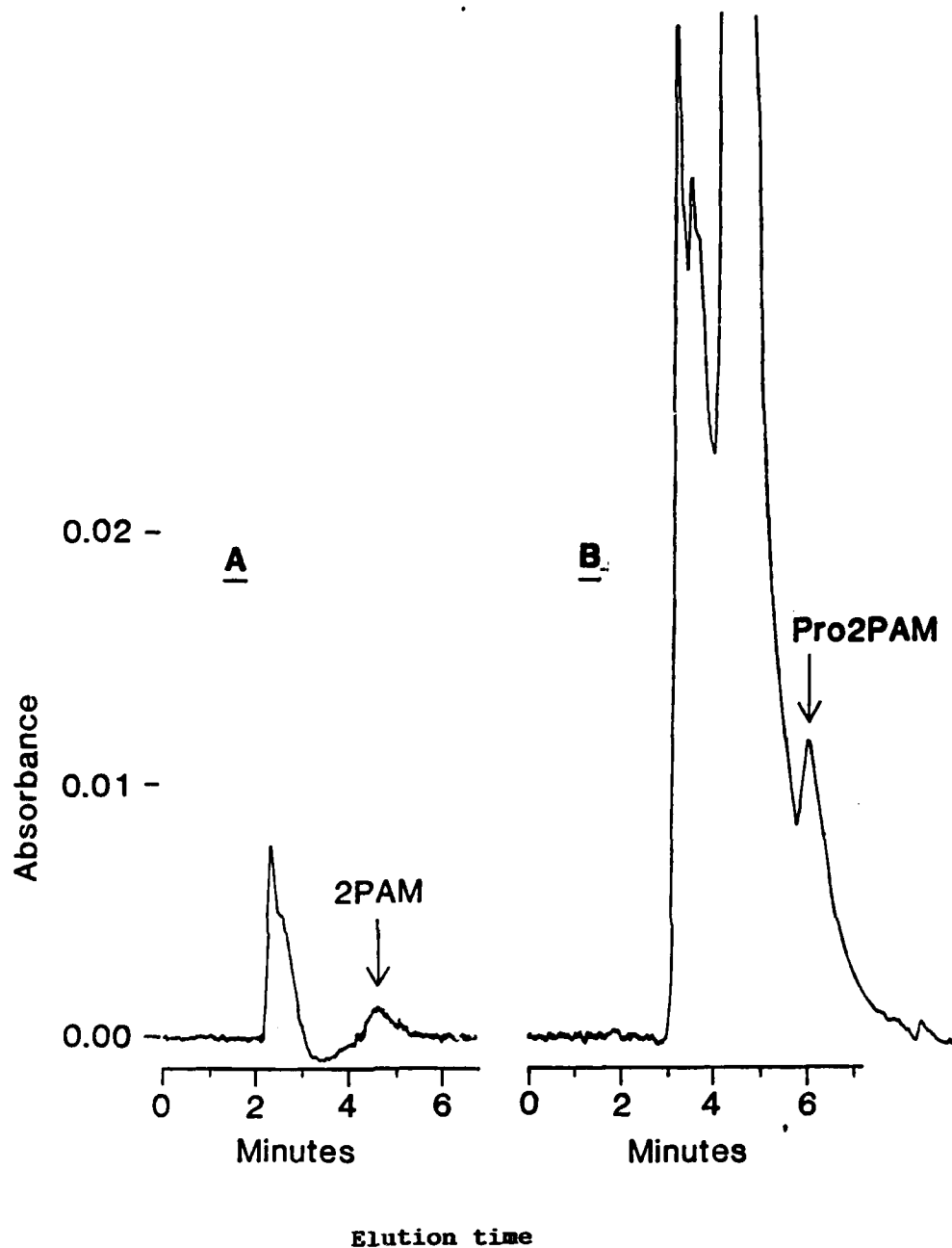


Figure 13. HPLC Elution Pattern of 2-PAM and Pro-2-PAM.

Typical elution profiles of 2-PAM (A) and pro-2-PAM (B) from a dose of pro-2-PAM (i.v., 50 mg/kg) in brain tissue samples, using an acidic solvent system (100% H_3PO_4 buffer, 0.1 M, pH = 2.5) and chromatography on a PRP-1 reversed phase column. 20 μ l injection; flow rate 1.0 ml/min; detector wavelength = 312 nm (A), 251 nm (B).

These HPLC systems were used to determine the CNS permeability of pro-2-PAM and the extent of its conversion to 2-PAM in the brain. Animals were given injections of either 2-PAM (i.m., 50 mg/kg) or pro-2-PAM (i.v., 50 mg/kg) and then sacrificed at various times up to 15 minutes after injection and the brain levels of 2-PAM or pro-2-PAM determined using the HPLC systems illustrated in Figures 12 and 13. Four important observations can be made about the data shown in Figure 14; (a) Two minutes after administration of pro-2-PAM, the ratio of pro-2-PAM to 2-PAM in the brain is approximately 2:7 (2.04 prodrug vs. 0.76 2-PAM % dose/gm brain); (b) 15 minutes after administration of the prodrug, there are still significant levels of pro-2-PAM (2.12% dose/gm brain); (c) administration of pro-2-PAM produces a higher brain level of 2-PAM (5 times) than that achieved with a comparable dose of 2-PAM; however, the magnitude of the increase is less than the 13-fold increase reported earlier by Shek *et al.*²; and (d) the combined brain levels of pro-2-PAM

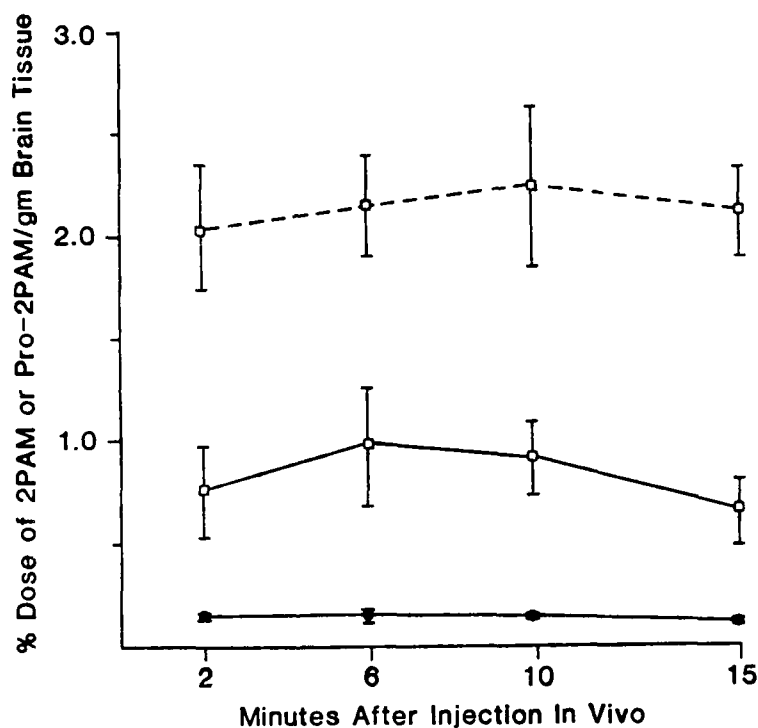


Figure 14. Brain Levels of 2-PAM and Pro-2-PAM.

Animals were given a 50 mg/kg dose of 2-PAM (i.m.) or pro-2-PAM (i.v.) and brain levels of 2-PAM and pro-2-PAM measured. 2-PAM injection: 2-PAM samples were analyzed in the basic solvent system (●—●, 0.1 M Na₂CO₃, pH = 10.5, 334 nm). Pro-2-PAM injection: Brain was quick-frozen and dissected in an acid bath to prevent conversion of pro-2-PAM to 2-PAM during analysis. Samples were analyzed in the acidic solvent system (0.1 M H₃PO₄, pH = 2.5) for pro-2-PAM remaining (□---□, 250 nm) and 2-PAM formed from pro-2-PAM (□—□, 312 nm).

and 2-PAM from a dose of the prodrug is 20 times higher at 2 minutes after drug administration than the brain level of 2-PAM from a dose of 2-PAM (2.8 vs. 0.14%, dose/gm brain).

Analysis of blood taken from the sacrificed animals showed that a dose of pro-2-PAM delivers 2-PAM mainly to RBC's, whereas a dose of 2-PAM is distributed mainly in the plasma (Figure 15). These results are consistent with those reported earlier by Shek *et al.*²³

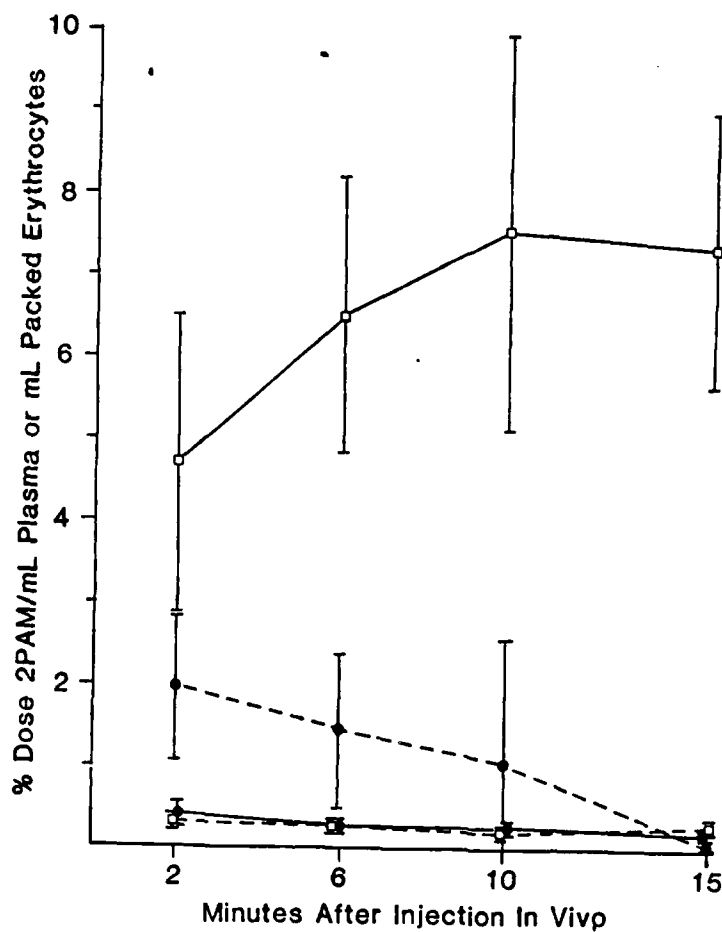


Figure 15. RBC and Plasma Levels of 2-PAM and Pro-2-PAM.

Animals were given a 50 mg/kg dose of 2-PAM (i.m.) or pro-2-PAM (i.v.) and RBC and plasma levels of 2-PAM and pro-2-PAM measured. 2-PAM injection: RBC (●—●) and plasma (●---●). Pro-2-PAM injection: RBC (□—□) and plasma (□---□). All samples were analyzed in basic solvent system (0.1 M Na₂CO₃, pH = 10.5, 334 nm).

Analysis of the kidneys from sacrificed animals showed that at 2 minutes the kidney level of 2-PAM from a dose of 2-PAM was significantly higher than the level of 2-PAM from a dose of pro-2-PAM (Figure 16). By 6 minutes the kidney levels of 2-PAM from the two dosage forms were essentially equivalent.

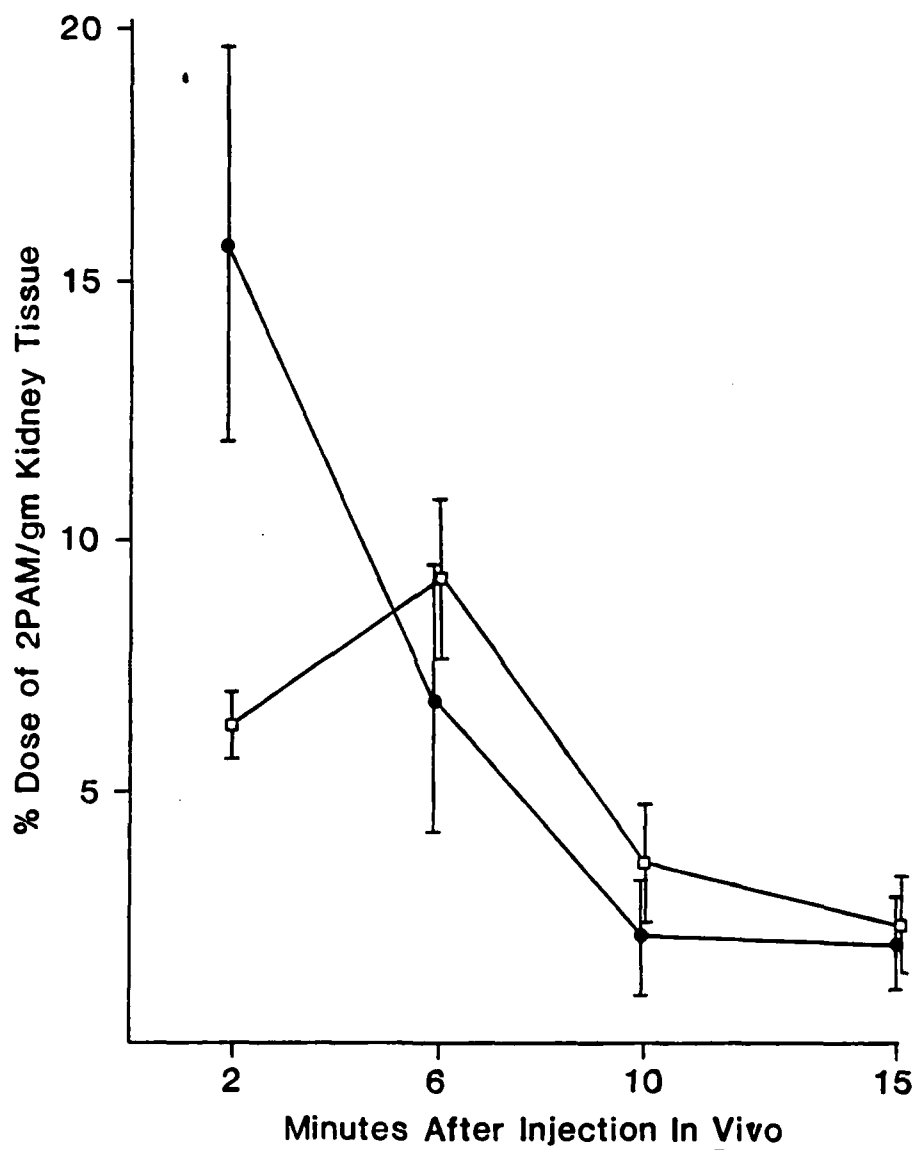


Figure 16. Kidney Levels of 2-PAM and Pro-2-PAM.

Animals were given a 50 mg/kg dose of 2-PAM (i.m.) and pro-2-PAM (i.v.) and kidney levels of 2-PAM and pro-2-PAM measured. 2-PAM injection (●—●) and pro-2-PAM injection (□—□) were both analyzed as 2-PAM in basic solvent system (0.1 M Na₂CO₃, pH = 334 nm).

In order to explain the apparent stability of pro-2-PAM in brain tissue (Figure 19), we studied the oxidation of pro-2-PAM to 2-PAM in physiological buffers. As shown in Table 4, the rate of air oxidation of the prodrug to 2-PAM is inversely proportional to the initial concentration of pro-2-PAM. As shown in Table 5, the maximum percent conversion of the prodrug to 2-PAM in the presence of AgNO_3 is also inversely proportional to the initial concentration of pro-2-PAM.

Table 4. Rate of Pro-2-PAM Conversion to 2-PAM in a Physiological Buffer^a.

pro-2-PAM (M)	$t_{1/2}$ (min)
10^{-5}	3.2
10^{-4}	10.0
10^{-3}	16.5
10^{-2}	31.3

^aExperiments were carried out in MOPS buffer (0.1 M, pH = 7.4) containing bovine serum (1/1). At various times perchloric acid was added to samples. The samples were immediately filtered and analyzed by HPLC, using the acidic buffer system (0.1 M H_3PO_4 , pH = 2.5). 2-PAM was detected at 312 nm.

Table 5. Extent of pro-2-PAM Conversion to 2-PAM In Vitro^a.

pro-2-PAM (M)	Maximum Conversion (%)
10^{-5}	86
10^{-4}	70
10^{-3}	41
5×10^{-2}	31

^apro-2-PAM samples were oxidized with excess AgNO_3 at pH > 7. After 20 minutes the samples were filtered and analyzed by HPLC, using the basic buffer system (0.1 M Na_2CO_3 , pH 10.5). 2-PAM was detected at 334 nm.

Based on the data shown in Tables 4 and 5, we predict that the percentage of dose per gram of brain tissue should be inversely proportional to the dose of pro-2-PAM administered. This hypothesis was confirmed by the data shown in Table 6. Although the brain levels of 2-PAM increased with increasing doses of pro-2-PAM, the percentage of dose in the brain is inversely proportional to the dose of the prodrug.

Table 6. Dependence of the 2-PAM Brain Levels on the Dose of Pro-2-PAM.

Pro-2-PAM Dose mg/kg	2-PAM Present in Brain ^a			
	2 Minutes		6 Minutes	
	µg/gm tissue	% dose/gm tissue	µg/gm tissue	% dose/gm tissue
100	32.6 (44.1-21.0) ²	0.94 (1.33-0.55)	46.9 (56.1-37.6)	1.33 (1.55-1.10)
50	26.1 (36.1-16.2)	1.54 (2.03-1.04)	28.8 (34.1-23.6)	1.75 (2.09-1.41)
10	7.1 (8.4-5.8)	1.90 (2.22-1.59)	6.8 (7.7-5.9)	1.91 (2.09-1.73)

^aGroups (n=3) of mice were given either 10, 50 or 100 mg/kg doses of pro-2-PAM (i.v.). The animals were sacrificed at 2 minutes or 6 minutes after drug administration. The brains were quickly frozen in a -70° C dry ice bath and dissected under acidic conditions to prevent conversion of the prodrug to 2-PAM. Samples were then analyzed by HPLC, using the acidic solvent system (0.1 M H₃PO₄, pH = 2.5). 2-PAM was detected at 312 nm.

^bConfidence limits.

Our data, as well as literature data (Table 7), suggest that 2-PAM is more effective than pro-2-PAM in protecting animals from an organo-phosphate challenge. These results are inconsistent with the observation that the prodrug produces a higher brain level of 2-PAM. As shown in Figure 19, the prodrug also produces elevated brain levels of pro-2-PAM, but this dihydropyridine oxime probably does not contribute to the reactivation of brain AChE. It is noteworthy that pro-2-PAM given prophylactically 10 minutes prior to organophosphate exposure (Table 7) shows a better protective effect than 2-PAM. These results might suggest that the $t_{1/2}$ for conversion of the prodrug to 2-PAM in biological tissues is longer than was previously anticipated, a result which is consistent with the data shown in Table 4.

Table 7. Literature Reports of the Protective Ability of Pro-2-PAM and 2-PAM against Organophosphate Challenge in Mice.

	2-PAM	pro-2-PAM	Ratio	Ref.
ED ₅₀ ^a (mg/kg)	7.9	20.3	2.5	b
95% confidence limit	(6.0-10.6)	(12.7-32.4)		
ED ₅₀ (mg/kg)	3.8	11.6	3.0	41 ^c
S.E.	± 0.8	± 3.0		
#Died/#Tested	0/6	3/6	--	42 ^d
Protective Ratio ^e (Oxime Administered Prophylactically)	1.41	2.20	0.64	29 ^f

^aDose required to obtain 50% survival at 24 hr.

^bPerformed in this lab: 2-PAM i.m., pro-2-PAM i.v.; vs. DFP (6.0 g/kg, s.c.).

^ci.p. injection of oximes; vs. paraoxon (0.9 mg/kg, s.c.).

^d34 mg/kg i.p. oxime dose; vs. paraoxon (8 μ mole s.c.).

^eProtective Ratio: LD₅₀ of prophylaxis and organophosphate/LD₅₀ of organophosphate.

^fmg/kg, i.m., oxime dose 10 minutes before DFP (s.c.) challenge.

E. Conclusions

1. Chemistry

- a) Various 5-substituted 2-PAM's (I, Br, Cl, CH₃, CN, CONH₂-substituted) have been synthesized and characterized.
- b) Various 3-substituted 2-PAM's (I, Br, Cl, CH₃-substituted) have been synthesized and characterized.
- c) Attempts to prepare the 3-cyano and 3-carboxamido 2-PAM's have been unsuccessful because of the inherent reactivity of these moieties and the possibility for intramolecular reactions with the neighboring oxime.
- d) The 3-iodo, 3-bromo and 3-chloro-2-PAM's were shown to be unstable in aqueous solution, apparently because of an intramolecular cyclization reaction involving the adjacent oxime.
- e) With the exception of 5-cyano-2-PAM (pK_a = 6.8), the 3- and 5-substituted 2-PAM's synthesized in this study had pK_a's for the oxime moieties in the optimal range (7.3-8.3) for reactivation of organophosphate-inactivated AChE.
- f) With the exception of 3-methyl and 5-methyl-2-PAM's, the 3- and 5-substituted 2-PAM's synthesized in this study had improved octanol/H₂O partition coefficients.
- g) Pro-2-PAM has been synthesized from 2-PAM using the procedures reported by Bodor et al.²¹ However, attempts to prepare various 5-substituted pro-2-PAM's (I, CN, CONH₂ substituted) have failed apparently because of the inherent chemical instability of the substituted dihydropyridinium oximes.
- h) In vitro studies showed that the rate and extent of pro-2-PAM oxidation to 2-PAM was dependent on the reaction conditions and the initial concentration of the dihydropyridinium species.

2. Biology

- a) When tested in vitro as regenerators of DFP-inactivated AChE, several of the 3- or 5-substituted 2-PAM's were more potent than 2-PAM. Order of potency: 3-chloro-2-PAM > 3-bromo-2-PAM > 3-methyl-2-PAM > 3-iodo-2-PAM > 5-methyl-2-PAM > 2-PAM > 5-chloro-2-PAM > 5-carboxamido-2-PAM > 5-bromo-2-PAM > 5-cyano-2-PAM > 5-iodo-2-PAM.

b) When tested in vivo for their ability to protect mice from a challenge dose ($2 \times LD_{50}$) of DFP, several of the 3- or 5-substituted 2-PAM's were more potent than 2-PAM. Order of potency: 5-iodo-2-PAM \gg 3-iodo-2-PAM $>$ 2-PAM $>$ 3-bromo-2-PAM = 5-bromo-2-PAM $>$ 5-carboxamido-2-PAM $>$ 5-methyl-2-PAM $>$ 3-methyl-2-PAM = 3-chloro-2-PAM = 5-chloro-2-PAM \gg 5-cyano-2-PAM.

c) When tested in vivo by WRAIR for their ability to protect mice from a challenge dose ($2 \times LD_{50}$) of soman, 5-iodo-2-PAM and 5-bromo-2-PAM were more potent than 2-PAM.

d) There does not appear to be a good correlation between a compound's ability to regenerate DFP-inactivated AChE in vitro and its ability to protect mice from exposures to DFP or soman in vivo. This may be due to inherent differences in the oxime's ability to regenerate eel AChE versus mouse AChE, or it may be due to differences in distribution and metabolism of the oximes in vivo.

e) Using a newly developed HPLC assay, 2-PAM and pro-2-PAM can be detected in brain and other biological tissues (e.g., kidney) and biological fluids (e.g., blood).

f) Pro-2-PAM can be detected in brain after administration of pro-2-PAM. Pro-2-PAM delivers more 2-PAM to the brain than a dose of the parent drug (2-PAM), but less than previously reported by Bodor *et al.*²¹ The differences are due to improved sample handling in our laboratory, by which we prevent the air oxidation of pro-2-PAM in biological samples during the workup procedure. This new methodology has led to a more accurate estimate of the brain levels of 2-PAM and pro-2-PAM.

g) The brain level of 2-PAM is proportional to the dose of pro-2-PAM administered. However, the higher the dose of the prodrug, the lower the percentage of the dose delivered to the brain.

F. Recommendations

1. Chemistry

a) Based on the results of our studies, structural modifications of 2-PAM could lead to more efficacious regenerators of AChE. Additional 3- or 5-substituted 2-PAM's should be synthesized in order to establish a predictable structure-activity relationship for this series of compounds.

b) The dihydropyridinium oxime approach to prodrugs of 2-PAM appears to be of limited therapeutic utility. Therefore, further synthetic efforts in this area do not appear warranted.

2. Biology

- a) Based on the results obtained to date with 5-iodo-2-PAM and 5-bromo-2-PAM, more extensive pharmacological and toxicological studies should be conducted with these analogs so as to evaluate their therapeutic potential.
- b) Other immobilized forms of AChE (e.g., human RBC, mouse brain) should be evaluated as in vitro screening assays for potential AChE regenerators. Additional effort is needed in this area in order to arrive at a more accurate in vitro predictor of the potential efficacy of a regenerator.
- c) Additional effort should be directed toward studying the biodistribution in vivo of pyridinium oximes.
- d) Additional effort should be directed toward studying how pyridinium oximes permeate biological membranes (e.g., blood brain barrier).
- e) Results of the studies outlined above should lead to the rational design of regenerators of AChE.

G. Experimental Methods

1. Equipment and Reagents

Electron impact mass spectra were recorded on either a Varian-MAT CH-5 or a Riber R-10-10 mass spectrometer with RDS data system for computer analysis and spectra printout. NMR spectra were obtained with either a Varian T-60, Hitachi Perkin-Elmer R-24B or Varian FT-80a and were run in 1% TMS/ CDCl_3 unless otherwise noted. The IR spectra were obtained on either a Beckman IR-33 or an AccuLab-4 spectrometer and samples were run as either neat films or KBr pellets (1:100). UV-vis spectra were recorded on either a Cary 219 or a Beckman DU-5 spectrophotometer. HPLC determinations were performed on a Beckman 342 system (112 pumps, 420 controller and 340 organizer), a Kratos 769Z variable UV detector and a Spectra-Physics 4270 recording integrator. Separations were performed on the following reversed-phase columns: a 5 mm x 15 cm Ultrasphere 5 μm ODS RP, a 5 mm x 10 cm BrownLee Spheri-5 5 μm RP, and a 5 mm x 20 cm BrownLee PRP-1 10 μm RP, all with matching 3 cm guard columns. Normal phase analysis was performed on an Alltech 0.5 x 25 cm silica gel 10 μm column with 3 cm guard column and preparative scale purifications on an Alltech 1 x 25 cm RSIL 10 μm column with a 5 cm guard column. Ionization constants and pH adjustments were performed potentiometrically using an Orion Research model 399A pH meter equipped with a gel-filled combination electrode. Melting points were obtained as either capillary melting points (uncorrected) on a Thomas-Hoover apparatus or as micromelting points (corrected) on a Fisher-Johns melting point stage. The AChE assay was equipped with a Gilson Minipuls 2 variable speed peristaltic pump and a Gilson HM Holochrome variable UV-Vis flow detector.

The chemicals and solvents were generally reagent or HPLC (chromatography) grade unless purity was not crucial. Thin layer chromatography (TLC) was carried out on Analtech SG GHLF 250 μm , Analtech Woelm SGF 1000 μm or EM SG-60 F254 200 μm plates. Liquid chromatography (LC) sorbents were either Brinkman EM SG-60 70-230 mesh or Woelm silica 63-200 mesh. Acetylcholinesterase (electric eel organ) was obtained commercially purified from either Worthington (1000-1400 units/mg) or Boehringer Mannheim (1000 units/mg). Acetylthiocholine, DTNB, MOPS, DFP, diethyl-p-nitrophenylphosphate (paraoxon) and TMB-4 (4) were obtained from Sigma and were used without further purification. DFP degrades slowly, even when stored at 4°C, and was replenished with a fresh supply at regular intervals to insure consistency. The low density polyethylene beads (4mm, precision) were supplied by Precision Plastic Ball Co., Chicago, IL. MINA was purchased from Pfaltz & Bauer, but required purification to homo-geneity by LC (SG Woelm, 8% acetone/methylene chloride v/v). Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN, and are within 0.4% of theoretical values unless otherwise noted.

a. Ionization Constant Determinations

The general procedure required the accurate preparation of .005-.01 M solutions of pyridinium oximes. These oxime solutions were then acidified to pH 3 with 0.005 M hydrochloric acid and titrated with 0.005 M sodium hydroxide. The pH was monitored potentiometrically with a pH meter and combination electrode. Data plots of pH vs. volume of sodium hydroxide

added were constructed and pKa's read from titration inflection points.

Partition Coefficient Determinations

The partition coefficients were determined by taking a 1.5 ml solution of quaternary oxime (10^{-5} - 10^{-3} M) in either deionized water or 0.1 M MOPS (pH 7.4) and vortexing for 0.5 minute with an equal volume of octanol (spectrograde, presaturated with water). The phases were separated and clarified by centrifugation and the concentrations in octanol determined spectrophotometrically.

2. Chemistry

3-Bromo-2-picoline (13c) and 5-Bromo-2-picoline (13l)

The method of van der Does³⁷ was employed to prepare 3-bromo-2-picoline (13c) and 5-bromo-2-picoline (13l). Four 250 ml pressure vessels were each filled with 100 ml of 65% oleum, 22 ml (220 mmol) of 2-picoline and 10.5 ml (204 mmol) of Br₂. After 16 hours of reaction, followed by sodium carbonate neutralization and vacuum distillation, the yield was 88 g (63%) of a light yellow-green oil, b.p. 45-60° C, 3 mm. Literature b.p. 71-73°C, 14 mm.³⁷ Analytical samples of each isomer 13c and 13l were prepared by column chromatography (silica gel, load ratio 1:200, eluted with 10% ethyl acetate/methylene chloride v/v), R_f 0.25 (13c) and 0.30 (13l).
13c - oil; IR (film): 3040 cm⁻¹ (Ar-H), 2980 (CH₂-H); NMR: δ 2.58 (s, 3H, CH₃), 6.95 (dd, 1H, Ar5H), 7.73 (dd, 1H, Ar4H), 8.32 (dd, 1H, Ar6H), J_{4,5}=8Hz, J_{4,6}=2Hz (TMS/CCl₄).
13l - m. p. (uncorr) 29-31°C; IR (melt) 3040 cm⁻¹ (Ar-H), 2910 (CH₂-H); NMR: δ 2.42 (s, 3H, CH₃), 6.92 (d, 1H, Ar3H), 7.57 (dd, 1H, Ar4H), 8.43 (d, 1H, Ar6H) J_{3,4}=9Hz, J_{4,6}=2Hz (TMS/CCl₄).

3-Amino-2-picoline (20) and 5-Amino-2-picoline (21)

The method of van der Does³⁷ was employed to prepare 3-amino-2-picoline (20) and 5-amino-2-picoline (21). Three 210 ml stainless steel bombs were each filled with 100 ml of 28% aqueous NH₄OH, 2.8 g (11 mmol) of copper sulfate pentahydrate, and 15 g (87 mol) of the mixture of bromo-picolines 13c and 13l. The combined reactions produced 27 g (96%) of a brown oil which was carried on to the next step without further purification.

20 and 21 - brown oil; IR (film): 3320 and 3200 cm⁻¹ broad (NH-H).

3-Chloro-2-picoline (13d) and 5-Chloro-2-picoline (13e)

Following the method of Talik et al.³⁸, 1.0 g (9.3 mmol) of the aminopicoline mixture afforded 0.84 g (71%) of a clear, colorless oil consisting of 13d and 13e after steam distillation and without vacuum distillation. A pure sample of 13d was obtained through an alternate synthetic route.

13d - clear oil; IR (film): 3100 cm^{-1} (Ar-H), 2980 ($\text{CH}_2\text{-H}$); NMR: δ 2.54 (s, 3H, CH_3), 6.97 (dd, 1H, Ar5H), 7.51 (dd, 1H, Ar4H), 8.38 (dd, 1H, Ar6H), $J_{4,5} = 8 \text{ Hz}$, $J_{4,6} = 2 \text{ Hz}$, $J_{5,6} = 5 \text{ Hz}$ (TMS/ CCl_4).

6-Amino-3-chloro-2-picoline (23)

The procedure of Kress *et al.*⁴³ was used to prepare 6-amino-3-chloro-2-picoline (23). Starting with 27.0 g (250 mmol) of 6-amino-2-picoline (22), 30.3 g (85%) of 23 as a yellow oil was isolated and was used without chromatographic purification.

3-Chloro-6-hydroxy-2-picoline (24)

A modification of the method of Baumgarten *et al.*³⁹ was employed in the synthesis of 3-chloro-6-hydroxy-2-picoline (24). A 5.0 g (35 mmol) portion of 23 yielded 1.3 g (26%) of 24 as a colorless amorphous solid. 24 - colorless solid; IR (KBr): 3480 cm^{-1} (OH), 1700 (C=O); Anal: C, H, N.

3,6-Dichloro-2-picoline (25)

The method of Baumgarten *et al.*³⁹ was used to prepare 3,6-dichloro-2-picoline (25). A mixture of 1.3 g (9.1 mmol) of phosphorous pentachloride was heated under reflux at 110-115° C in an oil bath for 3 hours. After cooling, 0.26 ml (2.8 mmol) of phosphorous oxychloride and 0.25 g (1.2 mmol) of phosphorous pentachloride were added and the mixture was refluxed for an additional hour. After cooling, the mixture was neutralized with saturated sodium carbonate and extracted with 50 ml of methylene chloride. The methylene chloride was evaporated and the residue was loaded onto a short silica gel column (25 g). After elution with ethyl acetate, the solvent was evaporated, leaving 0.96 g (64%) of 25 as a brown oil.

25 - brown oil; IR (film): 3100 cm^{-1} (Ar-H), 2980 ($\text{CH}_2\text{-H}$); NMR: δ 2.57 (s, 3H, CH_3), 7.12 (d, 1H, Ar5H), 7.58 (d, 1H, Ar4H), $J_{4,5} = 9 \text{ Hz}$ (TMS/ CDCl_3); MS: m/e 160 ($\text{M}^+\text{-H}$), 126 ($\text{M}^+\text{-Cl}$).

3-Chloro-2-picoline (13d)

A modification of the procedure of Baumgarten *et al.*³⁹ was employed to prepare 3-chloro-2-picoline (13d). A 0.50 g (3.1 mmol) portion of 25 was reacted to give 0.16 g (41%) of a lightly colored oil. The modification of the Baumgarten *et al.*³⁹ procedure included neutralization with saturated aqueous sodium carbonate instead of sodium hydroxide, extraction with ethyl ether instead of benzene, and exclusion of the charcoal step. Physical and spectral data are reported in syntheses of 13d and 13e.

3-Bromo-2-picolinealdehyde (14c) and 5-Bromo-2-picolinealdehyde (14m)

Employing a modification of the method of Markovac *et al.*⁴⁴, 10.1 g (58 mmol) of unresolved 13c and 13m was mixed with 14.8 g of freshly ground iodine. The resulting complex was warmed until homogeneity was achieved and after cooling, the solidified complex was dissolved in 58 ml of DMSO. The dark solution was then stirred in a 150-160° C oil bath for

40 minutes, during which time evolving dimethyl sulfide was distilled away from the reaction through a distillation head and condenser. After cooling, the pH of the reaction was adjusted to 6-7 with a saturated sodium carbonate solution. The mixture was then exhaustively extracted with ethyl ether. The ether extracts were washed with brine, dried with sodium sulfate, filtered and flashed. The residue was then purified by column chromatography (silica gel, loaded in methylene chloride and eluted with 10% v/v ethyl acetate/methylene chloride) to afford 1.1 g (10%) 14c, 2.2 g (20%) 14m and 3.2 g (30%) of the mixture.

14c - yellow needles, m.p. (corr) 64-65°C; IR (KBr): 3040 cm^{-1} (Ar-H), 2810 (CHO), 1720 (C=O); NMR: δ 7.50 (dd, 1H, Ar5H), 8.15 (dd, 1H, Ar4H), 8.75 (dd, 1H, Ar6H), 10.03 (s, 1H, CHO), $J_{4,5} = 9$ Hz, $J_{4,6} = 2$ Hz, $J_{5,6} = 5$ Hz (TMS/ CCl_4/d_6 -acetone); MS: m/e 187, (M^+), 159, 157 ($\text{M}^+ - \text{CO}$), 78 ($\text{M}^+ - \text{Br}$, CO); Anal: C, H, N.

14m - yellow needles, m.p. (corr) 90-93°C; IR (KBr): 3050 cm^{-1} (Ar-H), 2850 (CHO), 1700 (C=O); NMR: δ 7.75 (d, 1H, Ar3H), 7.97 (dd, 1H, Ar4H), 8.78 (d, 1H, Ar6H), 9.92 (s, 1H, CHO), $J_{3,4} = 9$ Hz, $J_{4,6} = 2$ Hz (TMS/ CCl_4); MS; m/e 187, 185 (M^+), 159, 157 ($\text{M}^+ - \text{CO}$), 78 ($\text{M}^+ - \text{Br}$, CO); Anal: C, H, N.

3-Chloro-2-picolinaldehyde (14d) and 5-Chloro-2-picolinaldehyde (14e)

Preparation of 14d and 14e was as described for 14c and 14m. A 4.3 g (33.7 mmol) portion of 13d and 13e yielded, after chromatography, 0.67 g (14%) of 14d, 1.1 g (24%) of 14e and 0.95 g (20%) of the mixture.

14d - colorless needles, m.p. (uncorr) 45-45°C; IR (film): 2900 cm^{-1} (CHO), 1700 cm^{-1} (C=O), NMR δ 7.48 (dd, 1H, Ar5H), 7.89 (dd, 1H, Ar4H), 8.73 (dd, 1H, Ar6H), 10.26 (s, 1H, CHO) (TMS/ D_6 -DMSO/ CHCl_3); MS: m/e 141 (M^+), 113 ($\text{M}^+ - \text{CO}$), 78 ($\text{M}^+ - \text{COCl}$); (Anal: C, H, N.

14e - yellow solid; m.p. 58-61°C, literature^{4,5} m.p. 60-62°C; IR (film): 2900 cm^{-1} (CHO), 1740 (C=O); NMR δ 7.91 (m, 2H, Ar3, 4H), 8.79 (d, 1H, Ar6H), 10.09 (s, 1H, CHO) (TMS/ CHCl_3).

5-Cyano-2-picolinaldehyde (14g)

Preparation of 14g was as described for the bromo series, 14c and 14m. A 15.0 g (127 mmol) portion of 5-cyano-2-picoline (13g) yielded, on evaporation of the ether extracts, 12.6 g (75%) of a dark black oil which was then dissolved in methylene chloride and enough ethyl acetate to afford solution. This solution was then loaded onto a short 120 g silica gel column and 14g was eluted with methylene chloride. Evaporation of the methylene chloride left 11.6 g (69%) of a yellow amorphous solid. Yields ranged from 49-69%.

14g - yellow amorphous solid, m.p. (uncorr) 65-85°C, sublimes at 55°C, 15 mm; IR (KBr): 2860 cm^{-1} (CHO), 2225 (C=N), 1710 (C=O); NMR: δ 8.04 (m, 2H, Ar3, 4H), 9.01 (d, 1H, Ar6H), 10.07 (s, 1H, CHO), $J_{4,6} = 2$ Hz (14%) of 14d, 1.1 g (24%) of 14e and 0.95 g (20%) of the mixture.

3-Iodo-2-pyridinealdehyde diethyl acetal (26)

Employing a modification of Bodor *et al.*²¹, 1.22 g (5.2 mmol) of 3-iodo-picolinaldehyde 14m and 2.5 ml (15.0 mmol) triethylorthoformate were brought to a reflux under a nitrogen atmosphere in 10 ml of absolute ethanol saturated with HCl gas. The reaction was cooled to room temperature after 3-4 hours and nitrogen bubbled through the solution for 10-15 minutes to vent off excess HCl. The solution was transferred to a round-bottom flask and flashed to the ammonium salt. A 25 ml portion of saturated NaHCO₃ solution and 50 ml of ether were added to the residue and stirred. The ether layer was removed and washed with several small portions of water and with 2 X 15 ml of brine, dried over Na₂SO₄, filtered and flashed to a dark oil. The oil was loaded onto a short column of silical gel (70-230 mesh) and eluted with dry acetone. The resulting light tan oil was dried at 35-55 mm Hg for several hours, yielding 1.48 g (92% theoretical) of desired acetal 26.

26 - tan oil; NMR: δ 1.25 (t, 6H, CH₂CH₃) 3.7 (1, 4H, OCH₂CH₃) 5.75 (s, 1H, CHOCH₂), 6.95 (dd, 1H, Ar5H), 8.15 (dd, 1H, Ar4H), 8.6 (dd, 1H, Ar6H), J_{4,5} = 2Hz, J_{4,6} = 2Hz, J_{5,6} = 5Hz (TMS/CCl₄); MS: CI-NH₃, 308 (M⁺+1), 263 (M⁺+1-OCH₂CH₃).

3-Cyano-2-pyridinealdehyde diethylacetal (28)

Using the modified method of Friedman and Shecter⁴⁶, 1.4 g (4.6 mmol) of 26 and 0.41 g (4.6 mmol) of cuprous cyanide were brought to a vigorous reflux under a nitrogen atmosphere in 10 ml of dry dimethylformamide (DMF). The reaction went from colorless to deep red/brown over 6 hours. After cooling to room temperature, 80 ml of 20% aqueous sodium cyanide was added slowly with stirring to the reaction mix. The addition was exothermic as the copper complexes decomposed. The cooled mixture was extracted with several 20 ml portions of ether. Combined ether extracts were washed with 2 x 15 ml water and with 2 x 30 ml brine, dried over sodium sulfate, filtered and flashed to a deep red/brown oil. The oil was loaded onto a short silica gel column (25 g, 70-230 mesh) and eluted with 5% ethyl acetate/methylene chloride (v/v) to decolorize. The resulting light-tan oil, 0.42 g (44%), was single spot to TLC (SG, 5% ethyl acetate/methylene chloride).

30 - tan oil; IR: 2205 cm⁻¹ (CN); NMR: δ 1.25 (t, 6H, CH₂CH₃), 3.7 (dq, 4H, OCH₂CH₃), 5.65 (s, 1H, CHOCH₂), 7.35 (dd, 1H, Ar5H), 8.0 (dd, 1H, Ar4H), 8.75 (dd, 1H, Ar6H), J_{3,4} = 8Hz, J_{4,6} = 2Hz, J_{4,5} = 5Hz (TMS/CDCl₃); MS: CI-NH₃, m/e 207 (M⁺+1), 161 (M⁺+1 - HOCH₂CH₃).

3-Methyl-2-pyridine carbaldoxime (15a)

The method of Markovac *et al.*¹¹ was followed in the preparation of 3-methyl-2-pyridine carbaldoxime (15a). A 20 g (190 mmol) portion of 2,3-lutidine (13a) afforded 5.8 g (23%) of 15a as a colorless solid. 15a - colorless amorphous solid, m.p. (corr) 151-155° C, literature¹¹ m.p. 152-154° C.

5-Methyl-2-pyridine carbaldoxime (15b)

Preparation of the intermediate 5-methyl-2-pyridinealdehyde (14b) was as described for the bromoseries, 14c and 14m. Starting with 20.0 g (187 mmol) of 2,5-lutidine (13b), 12.7 g (56%) of crude 14b was obtained from the ethereal extracts. The aldehyde 14b was then dissolved in 20-30 ml of methanol and 12.0 g (173 mmol) of hydroxylamine hydrochloride (prepared as an aqueous solution neutralized with solid sodium carbonate) was then added to the methanolic solution. The mixture was then heated to boiling for 5-10 minutes and allowed to stand overnight. The solution was then concentrated on a hot plate and after cooling was extracted three times with 100 ml portions of ether. After concentration of the ether extracts, 15b was precipitated from solution with cyclohexane. Filtration yielded 2.4 g (9%) of 15b as an amorphous yellow solid.

15b - yellow solid, m.p. (uncorr) 156-158°C literature¹¹ m.p. 158-159°C.

3-Bromo-2-pyridine carbaldoxime (15c)

A saturated aqueous solution of 0.20 g (2.9 mmol) of hydroxylamine hydrochloride was neutralized with sodium carbonate and added to a methanolic solution of 0.38 g (2.0 mmol) of aldehyde 14c. The solution was warmed gently to boiling and allowed to cool with stirring overnight. The product was collected by suction filtration, the supernatant concentrated and a second crop of product collected. Recrystallization from ethanol afforded 0.37 g (90%) of 15c.

15c - tan needles, m.p. (corr) 231-234°C; IR: 3400 (OH), 3130-2760 (oxime); NMR: δ 7.32 (dd, 1H, Ar5H), 8.10 (dd, 1H, Ar4H), 8.42 (s, 1H, CHON), 8.61 (dd, 1H, Ar6H), 11.88 (s, 1H, NOH), $J_{4,5} = 8$ Hz, $J_{4,6} = 2$ Hz, $J_{5,6} = 5$ Hz (TMS/ d_6 -DMSO/ d_6 -Acetone); MS: m/e 202, 200 (M^+), 158, 156 (M^+ -CHNOH); Anal: C,H,N.

5-Bromo-2-pyridine carbaldoxime (15l)

Preparation of 15l was as described for 15c. A 0.41 g (2.2 mmol) portion of 14l was reacted with 0.25 g (3.6 mmol) of hydroxylamine hydrochloride to give 0.42 g (95%) of desired oxime 15l.

15l - tan needles, m.p. (corr) 209-211°C; IR: 3400 cm^{-1} (OH), 3100-2790 (oxime); NMR: δ 7.73 (d, 1H, Ar3H), 7.97 (dd, 1H, Ar4H), 8.03 (s, 1H, CHNO), 8.63 (d, 1H, Ar6H), 11.45 (s, 1H, NOH), $J_{3,4} = 9$ Hz, $J_{4,6} = 2$ Hz (TMS/ d_6 -DMSO/ d_6 acetone); MS: m/e 202/300 (M^+), 158, 156 (M^+ -CHNOH); Anal. C,H,N.

3-Chloro-2-pyridine carbaldoxime (15d)

Preparation of 15d was as described for 3-bromo-2-pyridinealdoxime (15c). A 0.84 g (5.9 mmol) portion of 14d reacted to yield 0.45 g (48%) of light-tan needles after standing 30 hours.

15d - light-tan needles, m.p. (corr) 213-215°C; IR (KBr): 1620 cm^{-1} (C=N); NMR: δ 7.40 (dd, 1H, Ar5H), 7.94 (dd, 1H, Ar4H), 8.57 (m, 2H, Ar6H and CHNOH), $J_{4,5} = 8$ Hz, $J_{4,6} = 2$ Hz, $J_{5,6} = 5$ Hz (TMS/ d_6 -DMSO/ d_4 -MeOH); MS: m/e 156 (M^+ -H₂O), 121 (M^+ -Cl), 103 (M^+ -Cl, H₂O), 91 (M^+ -Cl, NO); Anal: C,H,N.

5-Chloro-2-pyridine carbaldoxime (15e)

Preparation of 15e was as described for 15c. The reaction of 1.1 g (8.0 mmol) of 14e with 0.75 g (11.0 mmol) of hydroxylamine hydrochloride yielded 0.87 g (70%) of light-tan needles.

15e - light tan needles, m.p. (corr) 194-196° C, literature³⁸ m.p. 194-195°C; MS m/e 156 (M^+), 139 (M^+-OH), 126 (M^+-NO), 113 (M^+-CHNO).

3-Cyano-2-pyridine carbaldoxime (15f)

To a solution of 1.0 g (5.0 mmol) of 28 in 10 ml of acetone was added 5 ml of 2 N hydrochloric acid. This solution was refluxed for 1/2 hour and was then evaporated to about 1 ml. An aqueous solution of 0.34 g (4.9 mmol) of hydroxylamine hydrochloride was added to the concentrated solution. Precipitation of a tan granular solid occurred after stirring overnight. The yield of 15f was 71% (0.51 g).

15f - tan granular solid, m.p. (corr) 196-197° C (dec); IR (KBr): 2200 cm^{-1} (C=N), 1620 (C=N); NMR δ 7.52 (dd, 1H, Ar5H), 8.18 (m, 2H, Ar4H and CHNOH), 8.71 (dd, 1H, Ar6H), 10.19 (s, 1H, CHNOH), $J_{4,5} = 9$ Hz, $J_{4,6} = 2$ Hz, $J_{5,6} = 5$ Hz (TMS/ d_6 -DMSO/ d_6 -acetone).

5-Cyano-2-pyridine carbaldoxime (15g)

Preparation of 15g was as described for 15c. A filtered solution of 10.4 g (78.8 mmol) of 14g in methanol reacted with 6.12 g (88.1 mmol) of hydroxylamine hydrochloride to give 7.7 g (66%) of a yellow amorphous solid. Further precipitation was accomplished by adding 100 ml of water to the supernatant. Another 1.5 g (13%) of material was obtained in this manner. Yields ranged from 65-79%.

15g - yellow amorphous solid, m.p. (corr) 221-224° C; IR (KBr): 2200 cm^{-1} (C=N), 1600 (C=N); NMR: δ 8.10 (m, 3H, Ar3, 4H and CHNOH), 8.93 (d, 1H, Ar6H), 11.81 (s, 1H, CHNOH) (TMS/ d_6 DMSO/ d_6 -CDCl₃); MS: m/e 147 (M^+), 129 (M^+-H_2O), 117 (M^+-NO), 104 ($M^+-CHNOH$), 90 (M^+-NO, HCN), 76 ($M^+-HCN, CHNOH$); Anal: C, H, N.

Ethyl-2-carbaldoxime nicotinate (15h)

Preparation of the intermediate aldehyde was as described for 14c and 14m. Starting with 15 g (91 mmol) of ethyl-2-methyl nicotinate, 8.6 g (53%, 48 mmol) of the aldehyde was isolated. A solution of 3.3 g (47 mmol) of hydroxylamine hydrochloride dissolved in minimum water was added to a slightly less than saturated methanolic solution of the aldehyde (8.6 g, 48 mmol). Solid sodium carbonate was slowly added to bring the pH up to 6-7, at which point a gas evolved. The solution was allowed to stand overnight and the resulting precipitate was suction-filtered, yielding 3.8 g (22%) of a granular, yellowish solid.

15h - yellowish solid, m.p. (corr) 172-174° C; IR (KBr): 3420 cm^{-1} (OH), 1720 (C=O); NMR: δ 1.33 (t, 3H, CH₂CH₃), 4.32 (q, 2H, CH₂CH₃), 7.45 (dd, 1H, Ar5H), 8.06 (dd, 1H, Ar4H), 8.54 (s, 1H, CHNOH), 8.71 (dd, 1H, Ar6H), 11.71 (s, 1H, CHNOH), $J_{4,5} = 8$ Hz, $J_{4,6} = 2$ Hz, $J_{5,6} = 5$ Hz (TMS/ d_6 -DMSO/ d_6 -acetone); MS: m/e 194 (M^+), 149 ($M^+-OCH_2CH_3$), 131 ($M^+-OCH_2CH_3, H_2O$), 78 ($M^+-CO_2CH_2CH_3, CHNO$); Anal: C, H, N.

Methyl-6-carbaldoxime nicotinate (15i)

Compound 15i was prepared by combining the procedures for the bromoaldehydes, 14c and 14m, and 3-bromo-oxime 15c. An 8.0 g (56 mmol) portion of methyl-6-methylnicotinate yielded 3.9 g (39%) of a yellow solid.

15i - yellow granular solid, m.p. (corr) 187-190° C, literature¹¹ m.p. 191-192° C.

3-Carboxamido-2-pyridine carbaldoxime (15j)

The basic method of Markovac *et al.*¹¹ was followed in the preparation of 3-carboxamido-2-pyridine carbaldoxime (15j). Into 30 ml of 28% aqueous ammonium hydroxide was suspended 1.00 g (5.2 mmol) of 15h. The slurry was stirred and warmed to 40-50° C until the solid dissolved. The solvent was evaporated to dryness, yielding 0.85 g (quant) of a colorless solid. Recrystallization, sublimation or chromatography did not appreciably purify this compound.

15j - colorless solid, m.p. (corr) 159-166° C (dec); IR (KBr): 1610 cm⁻¹ (COHN₂), NMR; δ 7.51 (dd, 1H, Ar5H), 8.01 (dd, 1H, Ar4H), 8.64 (m, 2H, Ar6H and CHNOH), J_{4,5} = 8 Hz, J_{4,6} = 2 Hz, J_{5,6} = 5 Hz (TSP/D₂O); MS: m/e 166 (M⁺+H), 148 (M⁺-H₂O), 131 (M⁺-H₂O, NH₂), 105 (M⁺-H₂O, NH₂, CN), 91 (M⁺-CONH₂, NO), 77 (M⁺-CONH₂, CHNOH).

5-Carboxamido-2-pyridine carbaldoxime (15k)

Using the basic procedure of Markovac *et al.*¹¹, 3.5 g (19 mmol) of 15i was suspended in 100 ml of 28% aqueous ammonium hydroxide. The mixture was allowed to stir overnight, during which time the starting material dissolved. The solution was then evaporated to dryness and the residue was recrystallized out of ethanol/water with Norit decolorizing. The orange plates were suction-filtered to yield 2.4 g (75%).

15k - orange plates, m.p. (uncorr) 232-234° C, literature¹¹ m.p. 234-236° C.

3-Methyl-2-pyridine carbaldoxime methiodide (16a)

A modification of the method of Poziomek *et al.*⁴ was used to prepare 3-methyl-2-pyridine carbaldoxime methiodide (16a). A 5.0 g (37 mmol) portion of 15a was refluxed with 9.0 ml (150 mmol) of methyl iodide in 70 ml of methyl ethyl ketone for 18 hours, yielding 6.1 g (60%) of bright-yellow needles on suction filtration.

16a - bright-yellow needles, m.p. (uncorr) 202-203° C (dec), literature⁴ m.p. 201° C; IR (KBr) 1610 cm⁻¹ (C=N); NMR: δ 2.60 (s, 3H, Ar3CH₃), 4.37 (2, 3H, N-CH₃), 7.99 (dd, 1H, Ar5H), 8.65 (m, 3H, Ar4, 6H and CHNOH) (D₂O).

5-Methyl-2-pyridine carbaldoxime methiodide (16b)

The preparation of 16b was as described for 3-methyl-2-pyridine carbaldoxime methiodide (16a). A 2.0 g portion of 15b with 4.5 ml of methyl iodide in 100 ml of methyl ethyl ketone yielded 2.4 g (70%) of yellow needles.

16b - m.p. (corr) 145° C, literature¹¹ m.p. 155-156° C.

5-Chloro-2-pyridine carbaldoxime methiodide (16e)

A modification of the method of Poziomek *et al.*⁴⁷ was employed to prepare 5-chloro-2-pyridine carbaldoxime methiodide (16e). A 0.50 g (3.5 mmol) portion 15e, 10 ml of methyl ethyl ketone and 5.5 ml (88 mmol) of methyl iodide were combined into a pressure vessel. The head space of the vial was thoroughly purged with nitrogen. After sealing the vessel, it was placed in an 80° C oil bath for 42 hours. The bright yellow precipitate suction filtered upon cooling gave a yield of 0.55 g (58%). 16e - bright yellow solid; m.p. (corr) 205-208° C (dec.), literature¹¹ 203-204° C; IR (KBr): 1650 cm⁻¹ (C=N); NMR: δ 4.41 (s, 3H, CH₃), 8.47 (m, 2H, Ar3H, 4H), 8.70 (s, 1H, CHNOH), 9.09 (s, 1H, Ar6H) (D₂O/d₆-DMSO).

5-Carboxamido-2-pyridine carbaldoxime methiodide (16k)

Preparation of 16k was as described for 16e. Starting with 2.00 g (12.1 mmol) of 15k and 11.5 ml (185 mmol) of methyl iodide in 100 ml of methyl ethyl ketone, a yield of 82% (3.06 g) of yellow needles was afforded. Yields ranged from 70-82%.

16k - yellow needles, m.p. (corr) 178-197° C, literature¹¹ m.p. 169-170° C; IR (KBr): 1680 cm⁻¹ (C=O), 1620 (C=N); NMR: δ 4.50 (s, 3H, CH₃), 8.50 (d, 1H, Ar3H), 8.71 (s, 1H, CHNOH), 8.82 (dd, 1H, Ar4H), 9.25 (d, 1H, Ar6H) (TSP/D₂O).

3-Methyl-2-pyridine carbaldoxime methochloride (17a)

The basic procedure of Kondritzer *et al.*⁴⁸ was used. A Dowex-1X8 resin was prepared as the chloride form by first washing with methanol and then stirring 15 minutes with each of three portions of 1 N sodium chloride. The resin was then washed with water until the washings no longer gave a positive chloride test with 1% silver nitrate. The resin was then allowed to swell in water overnight. A solution of 1.0 g (3.6 mmol) of 16a in minimum water was stirred for 3 hours with 30 g (wet weight, 30 meq) of the resin. The resin was filtered off and extracted three times with water. The water extracts and filtrate were then flashed to dryness and the residue was recrystallized out of ethanol. Crystallization was initiated with the addition of ethyl acetate. The tan needles were filtered to yield 0.48 g (72%).

17a - tan needles, m.p. (corr) 205-208° C (dec); spectra were identical to 16a; Anal: C, H, N.

5-Methyl-2-pyridine carbaldoxime methochloride (17b)

Preparation of 17b was as for 17a above. A yield of 0.38 g (57%) of 17b was obtained from 1.0 g of 16b.

17b - tan needles, m.p. (corr) 196-199° C (dec.); spectra identical to 16b; Anal: (+ 1-H₂O): C, H, N.

3-Bromo-2-pyridine carbaldoxime methochloride (17c)

Using the method of Bell and Zalay⁴⁹, 3.0 ml of methyl toluenesulfonate was added to 0.50 g (2.5 mmol) of 15c. The slurry was heated with stirring in a 120-125° C oil bath just until the oxime totally dissolved; about 45 minutes. The solution was cooled and dissolved into about 25 ml of anhydrous methanol. The product was precipitated with anhydrous ether and the supernatant was decanted 4 hours later from a gummy, greenish precipitate. The precipitate was redissolved in anhydrous methanol, which was then added to a 15-20 ml methanolic slurry of Dowex-1 resin (prepared as the chloride form, as described for 17a and extensively washed with anhydrous methanol to exclude water). After stirring for 3 hours, the resin was filtered off and the methanol was flashed to a residue. Recrystallization of the residue from ethanol/ethyl acetate, as for 17a, yielded 0.19 g (30%) of 17c.

17c - green crystals, m.p. (corr) 202-204° C (dec); IR (KBr): 3480 cm⁻¹ broad (OH), 1620 (C=N); NMR: δ 4.44 (s, 3H, CH₃), 7.94 (dd, 1H, Ar5H), 8.54 (s, 1H, CHNOH), 8.88 (m, 2H, Ar4, 6H) (D₂O); Anal: C, H, N.

3-Chloro-2-pyridine carbaldoxime methochloride (17d)

Preparation of 17d was as described for 17c. A 0.20 g (1.3 mmol) portion of 15d reacted to yield 0.010 g (4%) of colorless crystals.

17d - colorless crystals, m.p. (corr) 209-211° C (dec); IR (KBr): 3460 cm⁻¹ broad (OH), 1640 (C=N); NMR: δ 4.43 (s, 3H, CH₃), 8.15 (dd, 1H, Ar5H), 8.75 (m, 3H, Ar4H, 6H and CHNOH) (TSP/D₂O); Anal: C, H, N.

5-Cyano-2-pyridine carbaldoxime methochloride (17g)

Preparation of 17g was as described for 17c. Starting with 4.0 (27 mmol) of 15g, a dark black oil and crystalline mixture was obtained. This mixture was extracted quickly with two 10 ml portions of absolute ethanol to decolorize the product. The light brown solid residue was recrystallized out of ethanol/ethyl acetate as for 17a. The yield was 1.0 g (19%) of light brown crystals.

17a - brown flakes, m.p. (corr) 223-225° C (dec); IR (KBr): 2220 cm⁻¹ (C=N), 1650 (C=N); NMR: δ 4.48 (s, 3H, CH₃), 8.78 (m, 3H, Ar3, 4H and CHNOH), 9.42 (d, 1H, Ar6H), J_{4,6} = 2 Hz (D₂O); Anal: C, H, N.

5-Carboxamido-2-pyridine carbaldoxime methochloride (17k)

Preparation of 17k was as described for 17a. The iodide salt, 3.06 g (10 mmol) of 16k was converted to 1.46 g (68%) of tan flakes.

17k - tan flakes, m.p. (corr) 216-218° C (dec); spectra were identical with that of 16k; Anal: C, H, N.

5-Chloro-2-pyridine carbaldoxime methochloride (17e)

Preparation of 17e was as described for 17a. A 53% yield of 17e was obtained from 0.55 g (1.8 mmol) of 16e.

17e - tan crystals, m.p. (corr) 205-208° C (dec); spectra were identical with that of 16e; Anal: C, H, N.

3. Bioassay

a. Immobilized Acetylcholinesterase Assay

Purified AChE (E.C.3.1.1.7, Electrophorus electricus) and human RBC AChE were immobilized, using a modification³⁶ of the technique reported by Ngo, Laidler and Yam⁴⁰. The modifications were as follows: Precision-ground, 4 mm spherical, low density polyethylene beads were used; all reactions and washes were conducted using an overhead mechanical stirrer with Teflon paddle; treatment of the beads with thionyl chloride, ethylenediamine and required rinses was carried out under a nitrogen atmosphere; the beads were treated with 10% glutaraldehyde for 3 hours with one solution change; the attachment of glutaraldehyde to the bead could be monitored for completeness by incubating the beads in the presence of DTNB, since any noncapped amino groups on the bead surface reacted with DTNB resulting in the generation of a chromophore which was monitored at 412 nm; the enzyme was loaded onto the beads in a 2 hour room temperature incubation; and MOPS buffer (0.1M, pH 7.8) replaced phosphate buffer throughout.

Beads containing immobilized enzyme were stored at -16° C in MOPS buffer containing 40% glycerin. The stability of the enzyme activity was effected by freeze-thaw cycles; therefore, the beads were frozen in small batches. The activity was stable for up to 4 months when stored as described. Enzyme activity per bead averaged 0.5 units/bead; however, significant bead-to-bead variation was observed. Under normal assay conditions (0.1 M MOPS buffer, pH 7.8, 37° C), 1.5-2%/h loss of enzyme activity was observed.

b. Measurement of AChE Activity

The enzyme activity assay was based on the Ellman technique²⁸. Acetylthiocholine (in 90% ethanol) and DTNB (in 95% ethanol) were added to 30 ml of MOPS buffer as above to give concentrations of 1×10^{-2} M and 5×10^{-4} M, respectively. Figure 17 is a schematic of the closed loop flow-through system employed in the assay. The peristaltic pump was routinely run at 5.8 ml/min and the column effluent monitored at 412 nm. The column was a polypropylene cylinder which contained immobilized enzyme beads packed with alternating glass beads which optimized surface area and flow characteristics. A solution in the flask containing substrate and chromogen was directed via switching valves through the column. The flask contents were cycled so that a stable baseline rate of enzyme activity was observed on the recorder trace. An excess of organophosphate (e.g., DFP, soman, paraoxon) was added and after all enzyme activity ceased (approximately 5 minutes), the system was flushed with fresh buffer via switching valves.

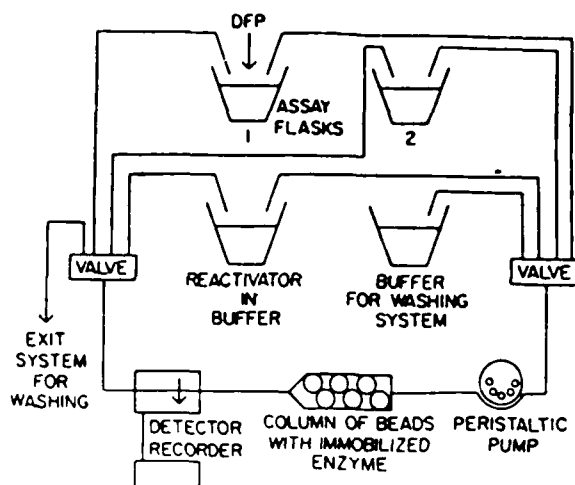


Figure 17. Schematic of Closed Loop, Flow-Through System for Immobilized Acetylcholinesterase Assay.

Flasks 1 and 2 contain substrate (ATC, 10^{-3} M) and chromogen (DTNB, 5×10^{-5} M) in MOPS buffer (0.1 M, pH 7.8) at 37°C. The peristaltic pump was run at a flow rate of 5.8 ml/minute and the detector was set at 412 nm.

Reactivators were routinely prepared in 200 ml volumes; however, some of the highest concentrations of 2-PAM and MINA were prepared in 100 ml volumes to conserve material. As mentioned earlier, phosphorylated oximes are potent enzyme inhibitors. To reduce the possibility of the phosphorylated oximes inhibiting the reactivated enzyme, the eluent was passed out of the system for the first 5 minutes of exposure to the reactivators. A fresh solution of reactivator was then recycled for the remaining exposure time. We observed no difference between the above procedure and pumping reactivator through the column in a single-pass fashion for the entire 45 minute exposure time. In the case of 2-PAM, we also examined the concentrations of reactivator (295 nm) via HPLC, both entering and exiting the column, and found no difference.

The detector output was recorded as absorbance units vs time which was converted to rate of substrate hydrolysis using the conversion of Ellman²⁸.

$$\frac{\Delta \text{Abs}/\text{min}}{1.36 \times 10^4} = \text{mol thiocholine/l.min.}$$

The substrate concentration routinely used in the assay was at saturation level, and the flow rate was an intermediate value. There was no apparent effect of flow rate on the regeneration of DFP-inhibited immobilized enzyme.

c. In Vivo - Animal Survival Studies

Adult male mice (CFI outbred), 25-35 g, obtained from Sasco, Inc., Omaha, NE, were used. Experiments were performed, using the same shipment of animals for consistency. Freshly prepared DFP (6 mg/kg, $2 \times \text{LD}_{50}$) in water was injected s.c. (20-35 μl). Reactivators (up to 100 mg/kg) were prepared in water and injected into the left hind limb i.m. (100 μl , 1%

body volume), except for pro-2-PAM (8), which was administered i.v. (tail, 20 second infusion) in freshly prepared and deoxygenated citric acid buffer (50 mM, pH 3.5).²⁹ (For ease of use, i.m. injections were preferred. While i.m. and i.v. injections of 2-PAM did not significantly change biodistribution, pro-2-PAM had to be injected i.v. due to other studies.²⁹) Groups of four or five animals/dose and four dose levels were used. Survivors were counted at 24 hours.

d. HPLC Assay for Pyridinium Oximes

The parent drug, 2-PAM, was injected i.m. (50 mg/kg in water). (There were no significant differences in the brain levels of the drug after i.m. or i.v. administration.) The pro-2-PAM (50 mg/kg in 0.05 M citrate buffer, pH 3.0) was injected iv in the lateral tail vein, unless otherwise noted.

Animals were decapitated at the end of the time period. Blood was collected with 0.15% EDTA to prevent clotting. The sample was immediately centrifuged for 5 minutes at 1000 x g. Perchloric acid was added to the plasma sample to precipitate proteins and centrifuged for 10 minutes (1000 x g). The clear supernatant was filtered through a nitrocellulose filter (0.45 μ m), basified with NaOH and stored at -20° C until direct injection on the HPLC. One hundred μ l of the packed RBC were diluted 5-fold with water and frozen. Perchloric acid was added to the thawed sample to precipitate proteins and the supernatant was centrifuged, filtered and basified as above.

Organs were surgically removed, weighed and homogenized in a rotary homogenizer (Tekmar Tissumizer) for 15-45 seconds in 1 ml of water, except for brain samples analyzed for unconverted pro-2-PAM (see below). Concentrated perchloric acid was added and the sample centrifuged for 10-15 minutes (1000 x g). The clear supernatant was filtered (0.45 μ m), basified and stored as above until direct HPLC injection after equilibrating to room temperature.

It was noted that a strongly acidic medium stopped the conversion of pro-2-PAM to 2-PAM in the presence of oxygen or even a strong oxidant such as AgNO₃. Therefore, a sample could be analyzed with the unconverted pro-2-PAM still present. Also, it was found that the dissection of the brain and its exposure to the air seemed to increase the conversion process. Therefore, to analyze these brain samples for only the 2-PAM that was present in vivo at the moment of decapitation, the whole head was quickly immersed in a dry-ice bath of methylene chloride (-70° C). The brain was then dissected out while submerged in a strongly acidic buffer (0.3 M phosphate buffer, pH = 1.5). The tissue was weighed in the acid buffer and gently ground by hand with glass beads while in a sonication bath to avoid introducing excessive air. Perchloric acid was added and the sample was centrifuged and filtered as above. The samples were analyzed for pro-2-PAM immediately.

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